

Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory
Laboratory 11: Induction of GFP

In today's exercise, we will learn about the process of *induction*, that is, how bacterial cultures are "induced" to start producing a particular protein. Over the course of the semester we have amplified, cloned, and transferred the green fluorescent protein gene from *Aequorea victoria* to a strain of the bacterium *Escherichia coli*. We are now interested in expressing this protein, purifying it and performing some simple characterization procedures. To begin this process, we must have a means of expressing the GFP gene that has been inserted into our plasmid vector.

The "off/on" switch in our vector is a DNA sequence just upstream of our GFP insertion site known as a **T7 promoter**. T7 is the name of bacterial virus (bacteriophage). A promoter is a sequence of DNA that typically occurs just in front ("upstream") of a gene. It is the site recognized by enzymes that initiate RNA transcription (RNA polymerase and associated transcription factors). The enzyme that recognizes T7 promoters is known as **T7 RNA polymerase**. Expression of genes linked to T7 promoters is very efficient.

The T7 promoter thus provides the switch to start gene expression. The T7 RNA polymerase turns the switch on. One way to induce expression of GFP in our transformed bacterial cells would be to infect them with the T7 virus. Theoretically this could work, but it would be a cumbersome approach. To ease this process strains of *E. coli* have been engineered that possess the gene for the T7 RNA polymerase in their genomic DNA. We used just such a strain for our transformation experiments earlier this semester. The T7 RNA polymerase gene in the genomic DNA of our *E. coli* cells is under the control of a separate promoter, known as the *lac* promoter. Like the T7 promoter, the *lac* promoter serves as a DNA signal that specifically binds an RNA polymerase, allowing the transcription of RNA from genes linked to it. As you may have learned in genetics or cell biology *lac* promoters are, by default, turned off because they are normally bound by *repressor* proteins that prevent the binding of RNA polymerase. The repressor protein can be removed, however, if it comes into contact with the disaccharide *lactose* (milk sugar). We will use an analog of lactose, **IPTG** (isopropyl-beta-D-thiogalactopyranoside) to release the repressor from the *lac* promoter, allowing expression of T7 RNA polymerase.

Thus, our expression system consists of two components: a T7 RNA polymerase gene in the bacterial genome, which is under the control of a *lac* promoter, and a plasmid-bound GFP gene, which is under the control of a T7 promoter. Let's follow the sequence of events to turn on (*induce*) the expression of GFP (figure 1).

Adding IPTG to a cell culture turns on the *lac* promoter by binding to, and releasing, the repressor protein bound to the *lac* promoter. The release of the repressor allows the transcription and translation of the linked T7 RNA polymerase gene. The T7 RNA polymerase produced, in turn, recognizes the T7 promoter on the GFP-containing plasmid. This results in the transcription and translation of Green Fluorescent Protein.

The net result is that the addition of IPTG ultimately results in the *induction* of GFP production.

In today's lab, we will follow the expression of GFP over time. We will do this by adding IPTG to a culture of transformed *E.coli* cells. We will remove samples at timed intervals to follow its expression. You will also prepare a cell-free extract of GFP from a culture that has already been fully-induced. In our next laboratory period we will purify GFP from this cell-free extract using **gel permeation chromatography**. Later, we will utilize polyacrylamide gel electrophoresis (SDS-PAGE) to isolate our protein and estimate its molecular weight.

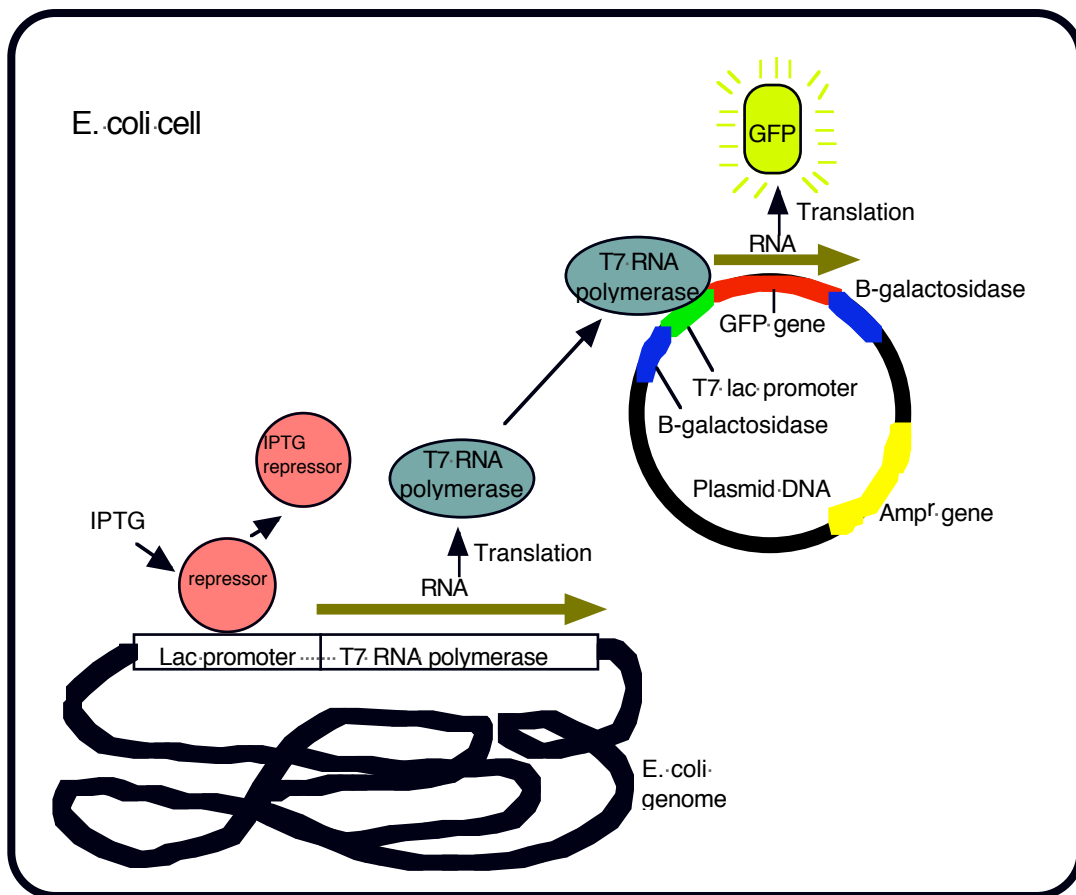


Figure 1. Induction of GFP gene by IPTG and T7 RNA polymerase

Materials & Reagents

5 ml of uninduced <i>E.coli</i> culture	50 ul 100 mM IPTG
200 ul of SDS loading dye	5 microcentrifuge tubes
50 ml centrifuge tube	Bacterial waste container (10% bleach)
transfer pipets	Dry ice/ethanol bath
3 ml Tris-EDTA (TE) buffer	Two 15 ml tubes w/caps
Microcentrifuge	balance
Allegra 21R centrifuge	30 ml of fully induced <i>E.coli</i> culture
15 ml “snap cap” tube	

Procedure

A. Induction of GFP

1. Transfer 5 ml of an *uninduced* bacterial culture to a 15 ml, “snap cap” tube. Label with your group’s initials. Add **45 ul of 100 mM IPTG**.
2. Label a microcentrifuge tube with your initials and a “0” to indicate a “zero” time point aliquot. **Transfer 0.5 ml of the culture from step 1** (containing the IPTG) to the microfuge tube. Return the “snap cap” tube to your instructor who will place it in the 37°C rotary incubator. Record the time.
3. Place the microfuge tube in the microcentrifuge and **centrifuge for 1 minute at maximum speed**. Transfer the *supernatant to a waste container (10% bleach)* on your bench (you may use a pipet to remove any residue). **Resuspend the bacterial pellet in 40 ul of SDS loading dye** as demonstrated by your instructor.
4. Remove additional 0.5 ml samples from the “snap cap” tube after 45 and 90 minutes. Treat each sample as in steps 2 and 3. Keep in mind that you won’t be analyzing these samples for another two class periods, so be sure to label each clearly with your initials, lab section and time point (0, 45, 90). When you have finished with your 90 minute sample, return all samples to your instructor for storage. In the meantime, proceed to part B.

B. Isolation of Cell-Free extract

The first step in any biochemical purification is to break open the cells containing the molecule of interest. We will accomplish this task today by repeatedly freezing and thawing a culture of fully induced bacteria. After the cells have burst, we will collect the GFP-containing supernatant by centrifugation. This supernatant represents a **cell-free extract** which we will use next week for gel permeation chromatography.

1. Add 30 ml of *fully induced* bacterial culture to a 50 ml centrifuge tube. Balance your sample with another group’s using the tabletop (non-enclosed) scale. Do this by placing your sample in a small beaker and zeroing the scale. Remove your sample and place the other group’s in the beaker. If the reading is negative, use a transfer pipet to add distilled water until the scale reads zero. If the reading is positive, zero the scale again and replace the other group’s sample with your own. The reading should be negative. Add water until the scale reads zero.

2. Place your sample in the centrifuge rotor (B/C F0850) across from your “partner” group, with whom you balanced your sample. When all groups have finished, your instructor will centrifuge the samples at 8,000 rpm (6900 x g) for 10 minutes.
3. Decant the supernatant from your sample into a bacterial waste container. Take care not to disturb the cell pellet.
4. Add 2 ml of TE buffer to your tube and resuspend the cell pellet by repeatedly pipetting up and down with a transfer pipet. Once the pellet is thoroughly resuspended, transfer the solution to a 15 ml, screw-cap tube. Mark the tube with your initials. Save your centrifuge tube for step 9.
5. Transfer the 15 ml screw-cap tube, containing your sample, to the dry ice/ethanol bath. Freeze for 3-5 minutes.
6. Thaw the cells in a 37°C water bath until they flow freely (3-5 minutes).
7. Repeat steps 5 and 6. This will lyse open the bacterial cells.
8. Transfer the lysed bacteria solution back to your 50 ml centrifuge tube. Balance your tube with another group’s as before.
9. Centrifuge (instructor) at 9500 rpm (9700 x g) for 10 minutes.
10. Carefully transfer the **supernatant** to a **new**, 15 ml, screw-cap tube. Mark with your initials, lab section and date.
11. Transfer 25 ul of this supernatant to a microcentrifuge tube. Add 25 ul SDS loading dye. Mark as “CFX” (cell-free extract) along with your initials.
12. You should now have four microcentrifuge tubes: the 0, 45 and 90 minute samples and the cell-free extract (CFX). You should also have your 15 ml screw-cap tube that has the GFP-containing supernatant. Be sure that all are labeled clearly. Store all samples as directed by your instructor.

Clean up

1. Empty bacterial waste containers down the sink
2. Wash centrifuge tubes and store, inverted, on the rack near the sink
3. Wash any dirty glassware

