PLANT BIOTECHNOLOGY AND PATHOLOGY (CH423)
Module Tutor: Dr. E. Smith

Practical 2

Part I: Investigation into the Regulation of expression of GFP
Part II: Purification of GFP using Column Chromatography

PRE-LAB QUESTIONS TO BE ANSWERED BEFORE PRACTICAL 2:

Write short notes on the words underlined in the background and introduction section (page 2 onwards).

Completing this exercise will aid you in preparing for your practical and in the summative assessment which you have to complete at the end of the 2 lab classes.

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Practical 2

Part I: Investigation into the Regulation of expression of GFP
Part II: Purification of GFP using Column Chromatography

21/10/02 and 28/10/02 (A602)
Dr. Helen Hooper, Ms. Karen Walker

Student Name _____ Mark _____ %

RISK ASSESSMENT AND SAFETY CONSIDERATIONS: you must read the following BEFORE you start the practical work.

Do not look directly at the UV light source or expose naked skin, doing so may result in damage to your eyes and skin. Wear lab coats and safety glasses.

Bacterial strain: E. coli HB101 are ACPD category 1 microbes and represent a minimal risk to individual lab workers if good laboratory practise (GLP) is observed. Wash hands with anti-microbial detergent if contaminated. Wipe up any spills with disinfectant.

All other reagents and chemicals- minimal hazard if GLP are observed.

Please Note:
(1) there are a set of **pre-lab questions** which may require **extra research and reading** that should be answered **before** you take the first practical class (and will help you to understand and complete the practical class and summative assessment).
(2) you are advised to read through this schedule before the first practical class.
(3) this practical has 2 parts and takes place over 2 weeks, you must therefore bring this schedule to **each** practical class.

Part I: Investigation into the Regulation of Expression of GFP

BACKGROUND AND INTRODUCTION

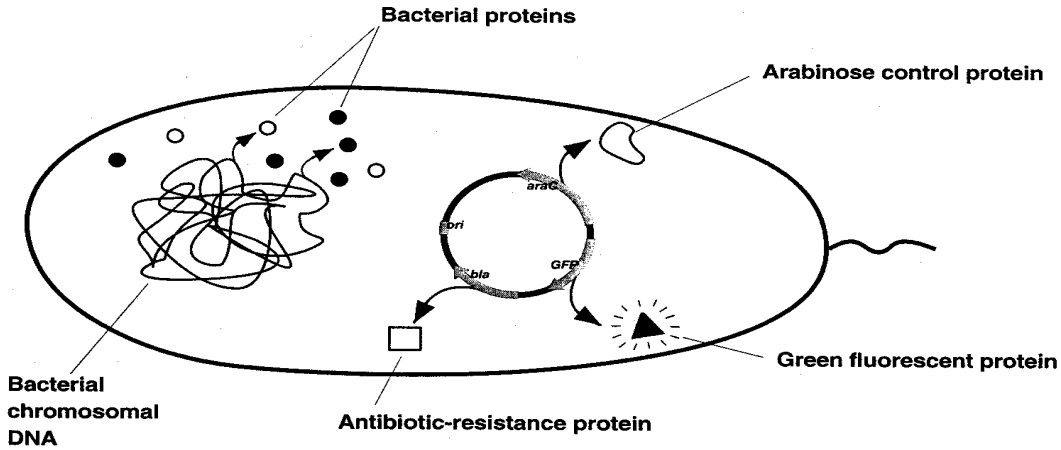
The **G**reen **F**luorescent **P**rotein (**GFP**) cDNA from the bioluminescent jellyfish *Aequorea victoria* has been cloned into a plasmid vector to create: **pGLO**. As the name might suggest, GFP fluoresces bright green under uv light. It can be viewed in real time in live cells; for this reason it is a valuable marker gene.

The GFP cDNA in pGLO is under the control of a regulatory DNA "switch" that allows its expression to be regulated. This switch is the arabinose operon promoter sequence ($\mathbf{p_{BAD}}$). In the arabinose operon a group of 3 clustered genes (***ara*B**, ***ara*A** and ***ara*D**) encode enzymes that metabolise arabinose. Expression of the 3 genes is dependent on initiation of transcription from the p_{BAD} promoter sequence. When arabinose is not present in the growth media an inhibitory protein (***ara*C**) binds to p_{BAD} and prevents transcription of the operon. When arabinose is present it binds to the *ara*C protein, causing a conformational change and initiation of transcription. When all the arabinose has been metabolised *ara*C returns to its original shape and transcription is shut off.

The DNA code of pGLO has been engineered to include the p_{BAD} promoter and the *ara*C gene. However, the arabinose operon has been replaced by the GFP gene; therefore in the presences of arabinose GFP is produced. In addition, pGLO also has an antibiotic resistance gene, which conveys a selective advantage to bacteria that maintain the plasmid when grown on antibiotic. This gene (***bla***) codes for an enzyme, β-lactamase, that confers resistance to the antibiotic ampicillin.

pGLO has been transformed into bacteria.

A schematic of a bacterium transformed with pGLOis given below:



Genetically engineered plasmid used to insert new genes into bacteria.

Green fluorescent protein.

Beta-lactamase. A protein which gives bacteria resistance to the antibiotic, ampicillin.

araC, a protein which regulates production of the green fluorescent protein.

This practical was developed as part of an LTSN Bioscience-funded project. Document downloaded from www.bioscience.heacademy.ac.uk/ftp/TDF/hooperprac.pdf

EXPERIMENTAL:

Work in SMALL GROUPS (but note that practical reports must be an individual effort!)

MATERIALS.

You are provided with:

Bacteria transformed with pGLO growing on an lb/amp/ara agar plate.

Bacteria transformed with pGLO growing on an lb/amp agar plate.

- lb agar plates
- lb/amp agar plates
- lb/ara agar plates
- lb/ara/amp agar plates
- Sterile toothpicks
- Inoculation loop
- Bunsen burner

The sterile technique for inoculation of both solid and liquid media will be demonstrated.

METHOD:

You are asked to use some, or all, of the materials listed above, to plan a series of experiments and controls to demonstrate (using colour under UV light) the regulation, activity and function of each of the following component parts of pGLO:

1: *ara*C gene
2: *bla* gene

Once you have decided upon your experimental plan, set up your experiments.

Preparation for part II of the practical.
Each group should inoculate a 5ml lb/ara/amp liquid culture with a green colony from the lb/ara/amp agar plate. In the second part of the practical you will be purifying the protein from this culture.

Note: *If you do not set this culture up, your group cannot perform the second part of the practical*!

Part II: Purification of GFP using Column Chromatography

Background and Introduction:

Column chromatography is a powerful technique commonly used in protein purification. It is the aim of this practical to isolate and purify the GFP from the liquid cultures of cloned bacterial colonies you initiated in the first part of this practical (22/10/01). You will use hydrophobic interaction chromatography (HIC) to achieve the purification and monitor the purification process using UV.

GFP has surfaces that are very hydrophobic. When placed in a buffer with a high salt content hydrophobic interactions are favoured and hydrophobic proteins will "stick" to the column matrix. In contrast, hydrophilic proteins will not interact with the matrix and flow through. By altering the concentration of salt in the buffer less hydrophobic proteins (which are less securely bound to the matrix) will be washed off. In this way GFP can be efficiently separated from other bacterial proteins.

Recording Information: Remember that the following purification of GFP by column chromatography can be (and should be) followed using UV light. Your own observations are important in write ups!

Practical instructions:

Examine the bacterial cultures you grew in the first part of this practical. Observe the colour of the colonies under UV light and record the information required for the write up.

1: Vortex your 2ml culture of pGLO transformed bacterial (from part I) to fully resuspend the cells and then centrifuge 2x1ml at top speed in a microfuge for 1min. Discard the supernatant. Briefly centrifuge (pulse) the tubes again and remove the last drops of supernatant. **Observe the cell pellets under UV?**

2: Fully **resuspend** one bacterial pellet in 250µl TE by pippetting and/or vortexing (until there are no clumps of the pellet left). Transfer the resuspended cells into the second pellet and vortex/pipette until fully resuspended (i.e. you have re-combined the pellets).

3: Cell lysis.
Add 100µl lysozyme and mix. Freeze the tube (either in dry ice or in the -80^{o}C freezer) Prepare the column (step 4 below) while the bacteria are freezing. Once your bacteria are frozen, thaw them (hand warmth is sufficient). *Repeat the freeze-thaw step once more*. **Observe the colour of the lysate under UV light.**

4: Column Preparation
Plug the bottom of a 1ml plastic syringe (the column) with a small amount of glass wool and then cap the bottom of a 1ml plastic syringe with parafilm. Place it in tube in the rack and add 0.5ml sepharose (column matrix). Allow the matrix to settle. *From this point on treat the column carefully, to prevent the matrix packing unevenly and air bubbles forming*.

Remove the parafilm 'cap' and allow the column buffer to drain off (if the column doesn't seem to be draining check that an airtight seal hasn't formed between your column and the collecting tube).

Transfer the column to a new collection tube. Carefully add 2 x 1ml equilibration buffer and allow to drain until the liquid reaches the top of the matrix.

At this point cap the top of the column with parafilm (it is important that the column matrix does not dry out completely). The column is now ready for use.

5: Centrifuge the bacteria for 5 min at top speed in a microfuge and transfer 300µl of the supernatant to a new tube. Recentrifuge and transfer 250µl of the supernatant to a new tube.

6: Add 250µl binding buffer to the supernatant and mix.

7: Carefully load 250µl supernatant in binding buffer onto the column and allow to drain into collecting tube 1. **Observe the column and flow through under UV light.**

8: Transfer the column into a new tube and add 250µl wash buffer. Collect the flow through in tube 2. **Observe the column and flow through under UV light.**

9: Transfer the column to a third tube and add 750 µl elution buffer and collect the flow through. **Observe the column and flow through under UV light.**

ASSESSMENT: ***Your write up MUST be word processed.***

DEADLINE: ***Refer to module guide. Please note that the deadline is that last possible date a piece of work may be handed in- you are more than welcome to submit work before the deadline.***

Part I:

Write up a concise and clear experimental plan, the predictions for the experiments you set up and your observed results (which you will collect at the start of part II). The majority of the marks will be awarded for discussion of the experimental plan and results obtained. (/50)

Part II:

i) Were you successful in isolating and purifying GFP from the cloned bacterial cells? Explain your answer. (/15)

ii) Briefly describe the basis for hydrophobic interaction chromatography. (/5)

iii) The following are the recipes for the four buffers used during the chromatographic purification of GFP from the hundreds of other bacterial proteins.

1: 2.0 M $(NH_4)_2SO_4$)
2: 10mM Tris.Cl pH 7.5, 1mM EDTA
3: 1.3 M $(NH_4)_2SO_4$)
4: 4.0 M $(NH_4)_2SO_4$)

Which recipe corresponds to the: equilibration buffer, binding buffer, wash buffer and elution buffer? Why? (/7.5)

iv) What are you doing when you freeze/thaw the bacterial cell pellet? Why is this an important step? (/2.5)

v) What types/kinds of proteins would you expect to see if you were to analyse samples of the column flow-through collected after:

a) loading the column
b) washing the column
c) eluting the column (/5)

vi) Name one other marker enzymes and list any advantages GFP has over it. (/2.5)

vii) List one other type of column chromatography used for purifying proteins and briefly describe the basis of purification. (/2.5)

viii) Research one paper in which GFP was used. Give its full citation and a brief abstract (100 word max). (/5)

ix) The GFP has been mutated to give a series of proteins with altered properties. These include: an enhanced GFP (eGFP) that glows 40x brighter than the wild type and yellow, blue and red fluorescent proteins. Concisely describe how and why these proteins were engineered. (/5)

References:

Chalfie, M *et al*., Green Fluorescent Protein as a Marker for Gene Expression. *Science*, **263**: 802 (1994)

Prasher, D. *et al*., Primary structure of the *Aequorea victoria* green- fluorescent protein. *Gene*. 111: 229-33 (1992)

Schleif R. (1994) Two positively regulated systens, *ara* and *mal*. In *E. coli* and *Salmonlla, Cellular and Molecular Biology*. F. Neidhardt (ed). ASM Press.

Biotechnology Explorer: The pGLO system: http://www.explorer.bio-rad.com

GFP resource page: http://www.biochem.mcw.edu/science_ed/Pages/gfp/index.html