Second half of the semester: Weeks 5-8

EXPRESSION, PURIFICATION, DETECTION AND FUNCTIONAL ANALYSIS OF A MUTANT T7 RNA POLYMERASE

GENERAL OVERVIEW

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product, which commonly corresponds to a protein. There are several steps in the gene expression process, including transcription, translation and some post-translational modifications. The second part of the semester involves a series of experiments to be performed with your transformant DH5α clone containing the recombinant plasmid vector with the T7 RNA polymerase insert, pTrcHisB/T7.

- You will first use your positive transformant cell line selected in laboratory class 4 to inoculate a liquid culture and induce the expression of the T7 RNA polymerase protein. Notice that your expressed protein constitutes a fusion or chimeric protein because the His tag had been merged at the N-terminal end of T7 RNA polymerase.
- Upon completion of the induction protocol, the cells will be harvested and lysed to prepare a total protein extract.
- Your fusion protein will be further purified by His tag affinity chromatography. Your fusion T7 RNA polymerase containing a His tag will be selectively retained within the column, and all other peptides will be eluted and collected in the flow-through fraction.
- You will then perform a Western analysis using an antibody that had been specifically raised against the His tag. This protocol is used to assess for the presence of a His tag on your fusion protein.
- You will finally verify whether your mutant fusion T7 RNA polymerase is still enzymatically active or not. This will be achieved by assessing RNA transcription in presence of your enzyme source and a DNA template. You will perform the enzyme assay of your mutant T7 RNA polymerase in parallel with a wild type enzyme source.

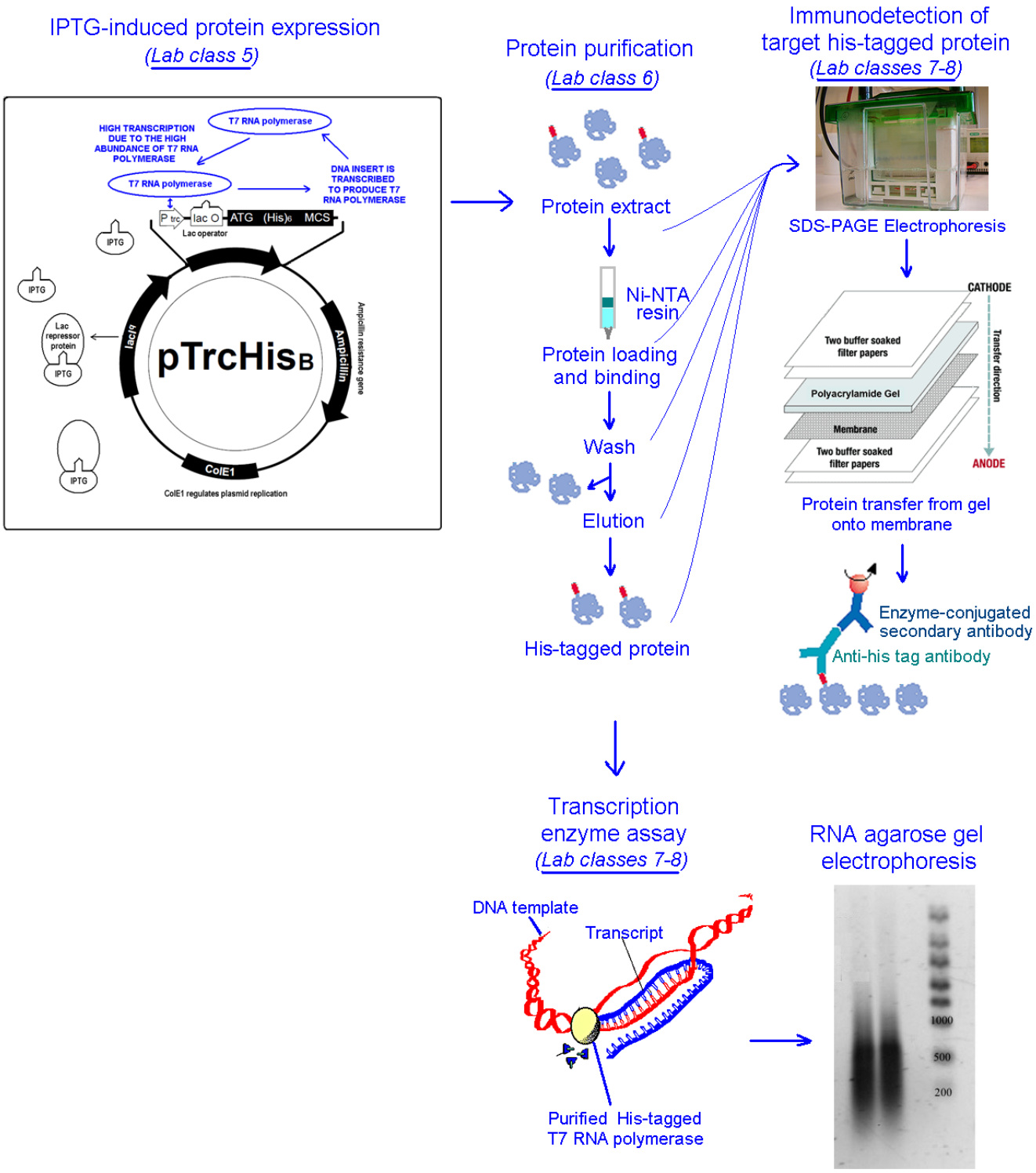
OVERALL TIMELINE

Week 5: Inoculation, induction and cell harvest

Week 6: His-tag protein purification by affinity chromatography

Week 7: First part of Western analysis and first part of enzyme assay

Week 8: Second part of Western analysis and second part of enzyme assay



Overview of experimental procedures for laboratory classes 5-8

Laboratory Class 5

PROTEIN EXPRESSION

LEARNING OBJECTIVES

Underlying molecular principles

- Explain the genetic components of the lac operon regulating the IPTG-inducible expression of T7 RNA polymerase in the TrcHisB/T7 and DH5α system

Hands-On Skills

- Inoculate and grow a transformant DH5α cell line
- Assess the cell growth phase by absorbance at 600nm
- Induce protein expression by adding IPTG
- Harvest cells from a liquid culture
- Prepare one buffer solution to be used in laboratory class 6

Analytical Skills

- No results to be generated this week

TO BE INDIVIDUALLY HANDED IN TO YOUR TA WHEN ENTERING THE LAB (/10 MARKS)

1. Perform a quick review of literature on recombinant protein expression in *E. coli* and indicate the protein yield reported in two research articles of your choice. Make sure you specify the units for the protein yield. For each article describe the nature of the protein that was overexpressed (membrane protein, cytosolic protein, …). Estimate how much of your recombinant T7 RNA polymerase enzyme you might expect from 50ml of liquid culture by assuming a yield comparable to the ones reported in the two articles you selected. A hard copy of each of your two articles along with your answer should be handed in to your TA when entering the lab. (/5 MARKS)

2. The overview figure provided in Experiment A shows the planning for the control aliquots to be assessed by SDS-PAGE in lab 6. Due to space limitation in the shaking incubator, some controls will be done collectively. Explain the information to be obtained from each of the two extra sets of controls to be done collectively:
    a. The inoculated 50ml culture containing 200ug/ml ampicillin is to be divided in two fractions of 25ml. At time zero of the induction, the first 25ml fraction is to be induced with 1mM IPTG whereas only water is to be added to the second 25ml fraction. A 1ml aliquot is to be withdrawn from each of the two fractions at both time zero and after a 1.5 hr incubation. (/2.5 MARKS)
    b. A liquid culture of non transformed DH5α cells will be incubated without ampicillin, and a 1ml aliquot is to be collected at time zero and the very end of the induction treatment with IPTG. (/2.5 MARKS)

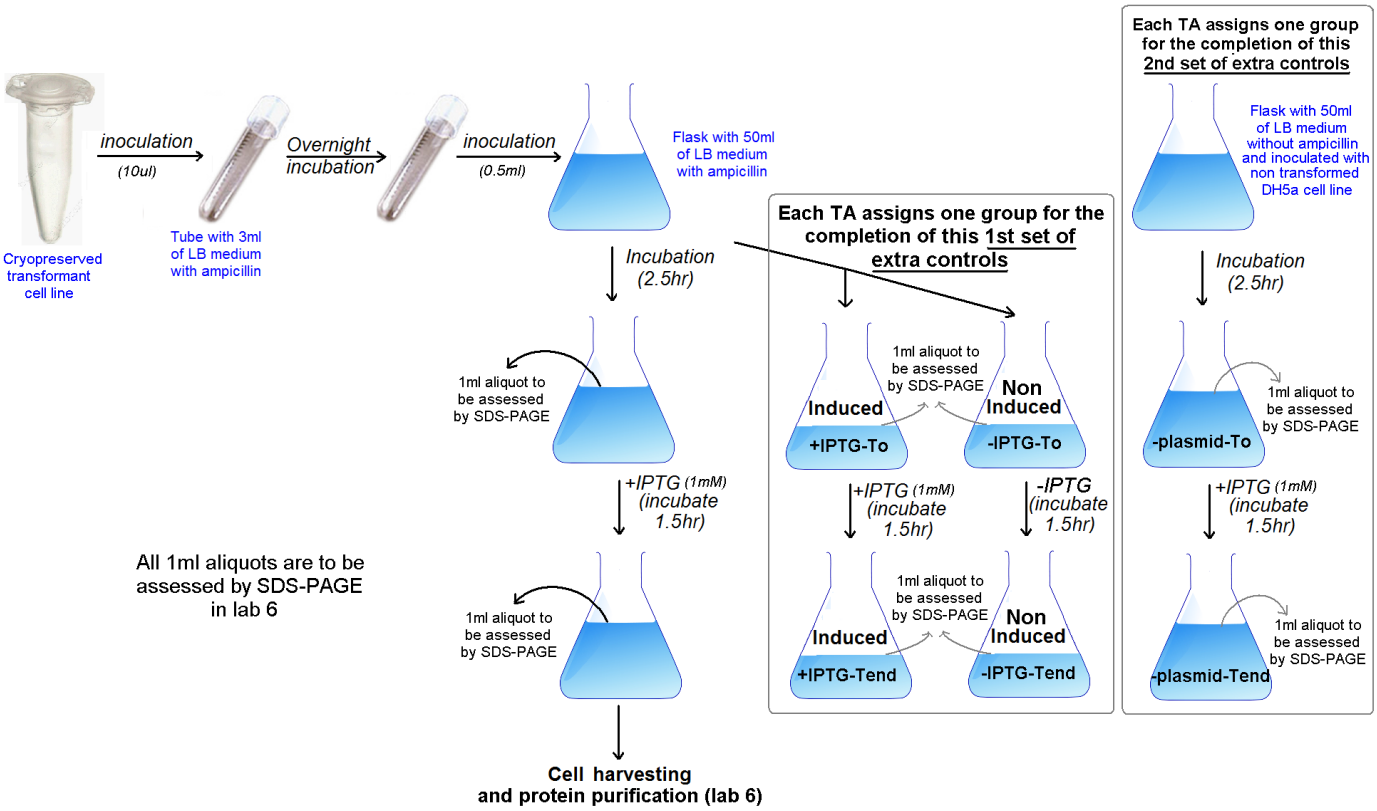
PROCEDURES

The present laboratory class involves extensive downtimes during which you will be required to prepare the buffer solutions to be used next week for the His tag affinity chromatography (see pre-assigned solutions below). The solutions will be put aside and returned to you by next week. The solutions will be shared by all students working under the supervision of the same TA. This means that the results of all your colleagues might be affected if you make a 'boo-boo' when preparing your solution. Be careful and ask your TA or someone else to verify your calculations.

- **Groups 1 and 9**
  - Prepare 60 ml of 8X Binding Buffer (4 M NaCl, 160 mM Tris-HCl, 80 mM imidazole, pH 7.9)
- **Groups 2 and 10**
  - Prepare 20ml of 8X Wash Buffer (4 M NaCl, 480 mM imidazole, 480 mM Tris-HCl, pH 7.9)
- **Groups 3 and 11**
  - Prepare 20ml of 4X Elute Buffer (1M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9)
- **Groups 4 and 12**
  - 4X Strip Buffer (2 M NaCl, 400 mM EDTA, 80 mMTris-HCl, pH7.9) prepare 10 ml
- **Groups 5-6 and 13-14**
  - 8X Charge Buffer(400 mM NiSO4) prepare 10 ml
  - Groups 5-6 and 13-14 work together to prepare one solution
- **Groups 7-8 and 14-15**
  - 1X T7 Storage Buffer (30 mM HEPES, 0.15M K-Acetate, 0.25 mM EDTA, 0.05% Tween 20, 1 mM DTT, pH 7.5) prepare 100 ml
  - Groups 7-8 and 14-15 work together to prepare one solution

EXPERIMENT A:

Step 1 is to be completed between 8-10:30am on the day preceding your regular lab class. Monday groups are to return to the lab on the preceding Thursday morning. You should plan about 20 min to complete the inoculation procedure.



1. Put your tube with your cryopreserved transformant cell line on ice until complete thawing. Then transfer 10ul of your cell culture into a snap-cap tube containing 3ml of liquid LB medium with 200ug/ml ampicillin. Put your inoculated tube in the shaking incubator at 37ºC.

2. Recover your inoculated snap cap tube from the incubator and transfer 0.5 ml of the cell suspension into a flask containing 50ml of liquid LB+AMP. Return your inoculated flask to the shaking incubator at 37ºC for 2 hours.

You can prepare the solution that was assigned to your group and to be used for the His tag affinity chromatography next week.

3. After 2 hr of growth, check the A_{600} of your cell culture. This can be done by swirling the flask gently to homogenize the content of the flask, tilting the flask to fill the sidearm with some liquid broth, and then inserting the sidearm into the aperture of the spectrophotometer to read the absorbance at 600nm. If the absorbance is below 0.25, return your flask in the incubator for another 30 min before verifying the absorbance again.

Culture flask with a sidearm

A reference aliquot (negative control) is to be put aside before proceeding to induction with IPTG. This initial aliquot is to be compared with a second aliquot to be sampled at the end of the induction with IPTG (positive control; see steps 6 and 8).

4. Transfer 1ml of the culture into a 1.5ml microfuge tube. This non induced aliquot is to be used for the preparation of a total protein extract at step 8.

5. Figure out the volume of a 100mM IPTG stock to be added to your culture to obtain a final concentration of 1mM. Add the IPTG to the flask and put it back in the shaking incubator at 37ºC for 2 hours.

6. Transfer 1ml of the culture into a 1.5ml microfuge tube. This IPTG-induced aliquot is to be used for the preparation of a total protein extract at step 8.

7. Transfer the content of your flask into a 50ml Nalgene centrifuge tube and centrifuge at 6,000g for 5 min. Discard the supernatant and freeze your pellet of cells at -20°C until next week.

Next week, only your IPTG-induced cell pellet is to be used for the purification of your His-tagged T7 RNA polymerase by His tag affinity chromatography. The other two 1ml aliquots put aside at steps 4 and 6 are to be kept for SDS-PAGE analysis to be done in lab 6.

8. Recuperate the two 1ml aliquots put aside at steps 4 and 6, and centrifuge them for 1min at 13,000rpm. Discard the supernatants and resuspend each cell pellet in 50ul of distilled water, which is a strong hypotonic environment triggering cell bursting and release of cytosolic proteins. Add 50ul of 2X Loading Buffer in each of your two tubes. These two aliquots are to be stored; you will recuperate those aliquots for SDS-PAGE analysis to be done in lab 7.

The extra two series of controls prepared by pre-designated students should be similarly mixed with the 2X loading buffer and returned to the TA. Those controls are to be assessed by SDS-PAGE next week, lab 7.

QUESTIONS/PROBLEMS

Underlying molecular principles

1. Explain the genetic components of the lac operon regulating the IPTG-inducible expression of T7 RNA polymerase in the pTrcHisB/T7 and DH5α system.

2. Define the concept of leaky expression, and explain the mechanism that might result in the leaky expression of T7 RNA polymerase in the pTrcHisB/T7 and DH5α system.

Analysis of results

3. Why was inoculation performed in two steps? It would have been more convenient to directly inoculate 50ml of broth on the day preceding the lab and immediately induce with IPTG at your arrival in the lab. Why were you asked to wait until the absorbance of the liquid broth at 600nm could reach a value of 0,25 before adding IPTG?

Reading materials for teaching topics

4. **Groups 7 and 15:** Any topic relevant to this lab can be chosen.

5. **Groups 8 and 16:** Any topic relevant to this lab can be chosen.