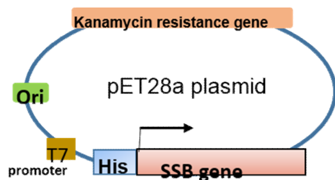
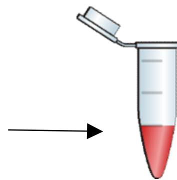
Week 4 – Expressing the SSB protein- Overview Contd.

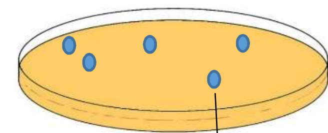
- Positive pET28a clones (from week 3) that carry the *SSB* gene insert will be tested for protein expression.
- The *SSB* gene in the pET28a plasmid is under the control of the T7 promoter. To activate transcription from the T7 promoter, cells carrying the pET28a plasmid need to express the bacteriophage-T7 RNA polymerase.
- BL21-DE3 cells are specially engineered bacterial cells that carry the gene for the T7 RNA polymerase. The T7 RNA polymerase gene in these cells is under the control of an inducible lac promoter.
- Therefore to activate expression of the SSB gene, the pET28a-SSB plasmid has to be transformed into the BL21-DE3 cells.

Step 1: Transform BL21-DE3 cells with pET28a-SSB plasmid



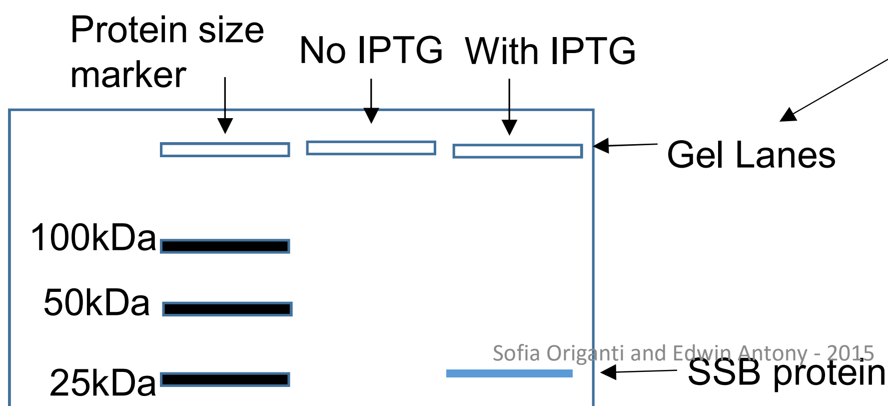


BL21-DE3 cells that take up the plasmid DNA will form colonies on LB-kan plates



Step 2: Grow BL21-DE3 colony in LB-kan media overnight and induce SSB expression by adding IPTG

Step 3: Lyse cells and check expression by SDS-PAGE



Week 4 – Expressing the SSB protein- Overview Cont'd.

The T7 RNA polymerase gene in the BL21-DE3 cells and the T7 promoter in the pET28a plasmid are under the control of the lac-inducible system.

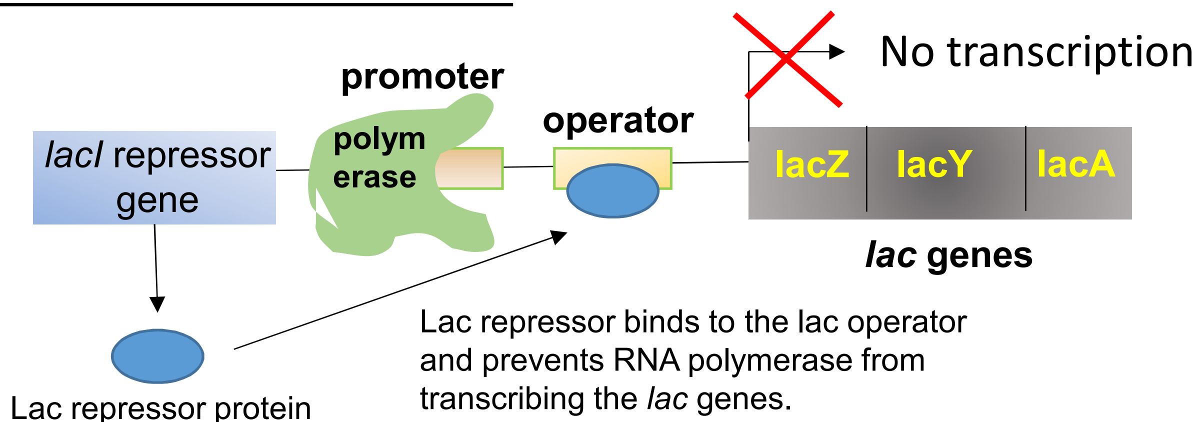
Lac operon-overview:

- The lac operon involves the expression of three *lac* genes essential for lactose metabolism as a single transcript transcribed from a single promoter. It is an inducible system wherein the presence of lactose induces the expression of the *lac* genes.

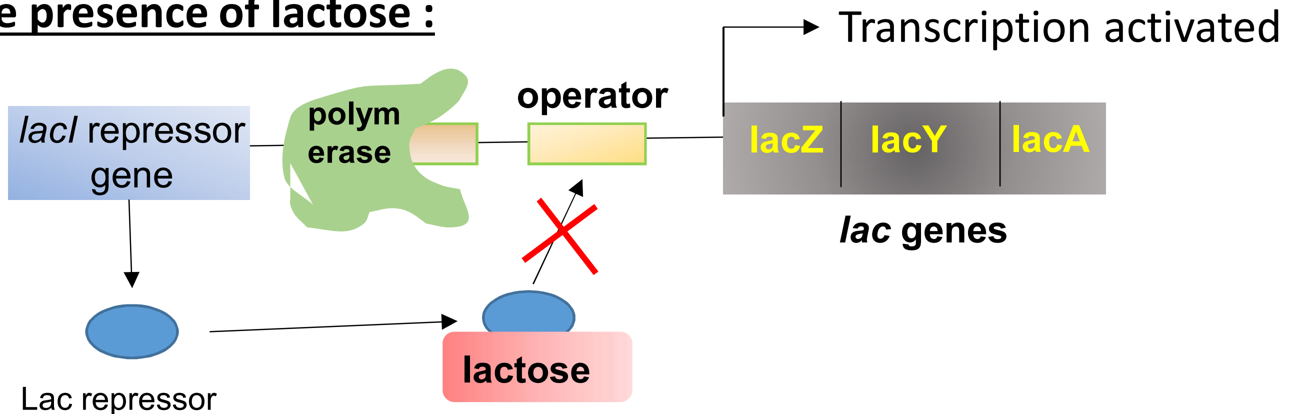
- In the absence of lactose, the expression of the *lac* genes is shut-off by the binding of the lac repressor protein to an operator sequence adjacent to the lac promoter. The lac repressor prevents the RNA polymerase from transcribing the *lac* genes.

- When lactose is present, lactose binds to the lac repressor and inactivates it. Inactive repressor cannot bind to the operator. This allows RNA polymerase to transcribe the *lac* genes.

In the absence of lactose :



In the presence of lactose :



Lactose binds to the lac repressor and inactivates it. This prevents the lac repressor from binding to the operator. RNA polymerase is now free to transcribe the *lac* genes

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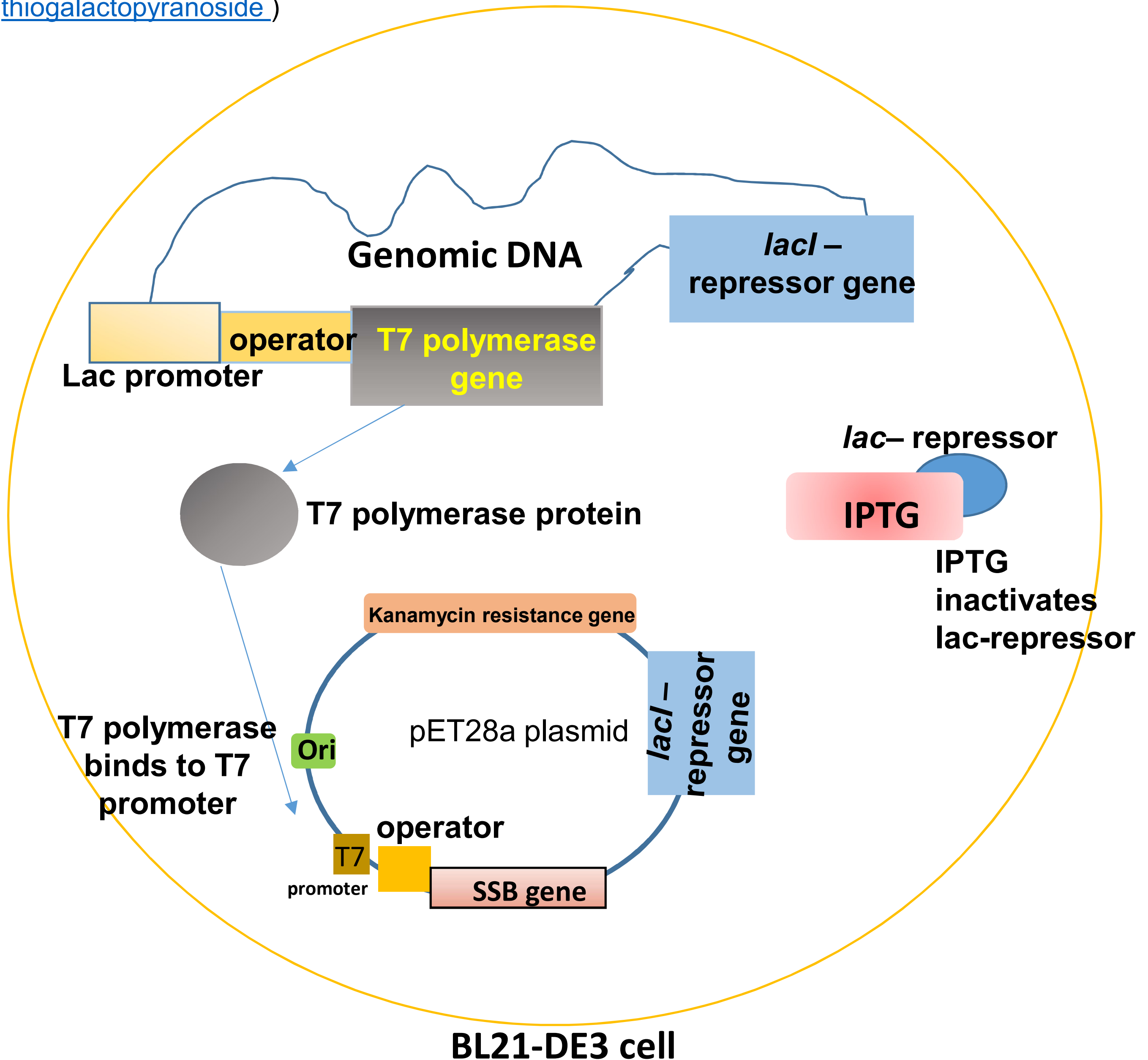
Week 4 – Expressing the SSB protein- Overview Cont'd.

Taking advantage of the lac-inducible system, the lac-promoter driven T7 RNA polymerase and the T7 promoter were engineered to be controlled by the addition of a stable lactose synthetic analog – IPTG (Isopropyl β-D-1-thiogalactopyranoside)



BL21-DE3 cell

- In the absence of IPTG (lactose analog), transcription from the lac promoter and any leaky transcription from the T7 promoter is shut-off by the binding of the lac repressor protein to an operator sequence adjacent to the lac and T7 promoter.

- When IPTG is added, it binds to the lac repressor and inactivates it. This activates transcription of the T7 RNA polymerase. T7 RNA polymerase binds to the T7 promoter. Since the T7 promoter is no longer inhibited by the lac repressor in the presence of IPTG, SSB expression is induced in the presence of IPTG. SSB gene is transcribed and translated to SSB protein.

Step I - Cell transformation protocol

1. Thaw BL21-DE3 cells **only on ice**.

2. Label a 1.5mL microcentrifuge tube with your gene name.

3. Add 50 uL of BL21-DE3 cells to the tube.

4. To the same tube, add 1uL of pET28a-SSB DNA.

5. Mix gently by tapping with fingers.

6. Incubate on ice for 5 min.

7. Heat shock by incubating tube at 42°C for 45 seconds.

8. Place back on ice for 2 minutes.

9. Add 250 uL LB-media.

10. Mix gently by tapping and help cells recover by shaking in incubator at 37°C for 15min at 220rpm.

11. Next to a flame (careful!), plate the entire reaction on a LB-Kanamycin plate-label with your group name and incubate the plate at 37°C overnight

(Note- Step 1- was completed before class)

Step 2 – Overnight Starter Culture

1. Prepare a 15-ml culture tube with 5 ml LB media containing Kanamycin (50 ug/ml final concentration).

2. Pick a colony from the plates transformed previous day (step 1) and grow overnight to generate a starter culture. Overnight incubation at 37°C with shaking at 220 rpm.

Step 3 – Induction with IPTG-protocol

1. Add 25 mL of starter culture to 1 L of LB-growth media in a 2.8 L fernbach flask. Label with your group name and incubate the flask at 37°C, shaking at 220 rpm.

2. After 5hrs, take 1ml of the culture in a cuvette and check if the cells reached 0.6 using a spectrophotometer set at wavelength of 600nM.

3. When cells reach OD 0.6, collect 1 ml culture sample in a 1.7mL micro centrifuge tube. Label with group name and as "No IPTG" . Spin this culture at 13000 rpm in a table top centrifuge, discard the supernatant and freeze the cell pellet at -20°C.

4. Add 400 ul of a 1 M IPTG stock (0.4 mM final) and continue shaking at 37°C at 220 rpm for 3 hrs (alternatively, this culture can be grown overnight at 20°C).

5. Remove the flask after 3 hrs and add the cell culture solution in 2- 500 mL bottles. Also, collect 1 ml culture sample in a 1.7mL micro centrifuge tube. Label with group name and as "with IPTG" . Spin this culture at 13000 rpm in a table top centrifuge, discard the supernatant and freeze the cell pellet at -20°C.

6. Collect cells by spinning in a centrifuge at 6000 rpm for 15 min at 4°C. Decant the solution and save the pellets at -20°C

7. Resuspend the " No IPTG" and "with IPTG" cells in 100 ul MQH2O by pipetting. Add 100 uL of 2X SDS protein -loading dye to the tubes and boil in a heat block for 5 min. Spin the tubes for 1 min at 13000 rom in a table top centrifuge and load 10 uL of the samples on an 12 % SDS-PAGE gel.

Please see the attached pdf file for background on SDS-PAGE and gel preparation protocol.

Step 4: Checking protein expression using SDS-PAGE protocol

Prepare the SDS-gel using the following reagents:

12% Resolving gel layer (8 mL total):

DD water – 2.7 mL
30% acrylamide – 3.2 mL
1.5 M Tris pH 8.8 – 2.0 mL
20% SDS – 40 uL
10% APS – 80 uL
*TEMED – 7.5 uL
(mix everything and add Temed last –right before pouring the gel)

4% Stacking gel layer (7.5 mL total):

DD water – 5.5 mL
30% acrylamide – 1 mL
1 M Tris pH 6.8 – 940 uL
20% SDS – 40 uL
10% APS – 80 uL
*TEMED – 7.5 uL
(mix everything and add Temed last –right before pouring the stacking gel layer)

Gel preparation:

- Clean the 1.5 mm glass plate and spacer plate with DD water and ethanol and wipe clean.
- Assemble the gel plates using the Bio-Rad gel assembly cassette.
- Pour 7.5 ml of the resolving gel layer. Immediately top the gel with isopropanol to ensure an even gel-loading layer.
- Wait for 20-30 min to polymerize.
- Decant the isopropanol and wash with dd water. Wipe down the assembly.
- Pour the stacking gel till it reaches the top of the gel assembly. Slowly insert a clean 1.5 mm comb (inserting the comb at a slight angle-helps!)
- Wait 20 more min

Loading samples and running the gel:

- Disassemble the gel assembly and keep the gel plates with the comb intact.
- Assemble the gel plates with the comb in the Bio-Rad gel loading cassette.
- Add 1L of 1X running buffer (100 mL of 10X running buffer + 900 mL ddwater). Load 10uL of sample in SDS-loading dye. Load protein size ladder.
- Run the gel at 120 milli amps for 1 hr.

Step 5. Coomassie Staining and destaining:

- Wash the gel cassette in ddwater to remove SDS buffer.
- Disassemble the gel cassette and add it to a plastic or glass dish.
- Add Coomassie stain-enough to cover the gel. Microwave on high for 20 sec and shake or rock at low speed for 15 min.
- Wash with dd water and add destaining solution enough to cover the gel.
- Crumple a Kim wipe and add it to the side of the dish (to soak up excess stain).
- Shake or rock gently for 1hr.
- Analyze gel bands using a light box.