Full Protocol 1
Protein-DNA Interactions

Overview

Estimated Experiment time: Three hours
Level: Undergraduate
Expected outcome: KD of Lac repressor (LacI, protein) / Lac operon (*lacO*, DNA).
Similar molecular systems: Binding between any kind of DNA operator and its associated regulator protein.

Background

The LacI/LacO/LacZ system is great to introduce students to basic molecular biology principles such as protein-molecule interactions (LacI and lactose), gene expression (*lacZ* expression in relation to LacI concentration), as well as protein-DNA interactions (LacI and *lacO*). Lac operon is a well-characterized example of transcription regulation.

The LacI protein is a negative regulator of the lac operon, repressing the expression of *lacZ*. Repression occurs through the binding of LacI to the DNA operator *lacO*, upstream of the *lacZ* promoter. However, lactose interacts with LacI and promotes a conformational change that destabilizes the LacI-*lacO* interaction.

Here we describe an experiment, using Surface Plasmon Resonance (SPR), designed for undergraduate students with the objective of measuring the dissociation constant (KD) for the LacI-*lacO* interaction.

Material

- Thiolated LacO (/5ThioMC6-D/TATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACA, 250 nM)
- Complementary LacO oligomer (500 nM)
- Running buffer: Phosphate Buffer Saline pH 7 (20mM phosphate, 50mM NaCl, 1mM EDTA)
- Regeneration buffer: PBS + BSA + 100 mM urea
- Sample buffer: PBS + 0.1 mg/mL BSA
- Destabilizing buffer: PBS + BSA + 0.05% SDS
- LacI purification from E. coli (optional, see appendix)
- Sample: LacI in PBS at 5 nM, 20 nM, 50 nM, 100 nM and 200 nM (monomer)
- MCH: 2 mM 6-mercaptho-1-hexanol in H2O
- SPR prism
- SPR fluidic cell

Protocol

1. Place a SPR prism and the SPR fluidic cell in the P4SPR.
2. Turn on the computer, open the P4SPR software, proceed with the initiation routine, and start recording your experiment (see appendix for detailed procedure).

General considerations

- *Avoid injecting air bubbles into the P4SPR channels.*
- *Beware that air bubbles can be introduced every time a syringe is placed on and removed from a port.*
- *After each injection, visually inspect if air bubbles are present on the chip's surface. If so, push the bubbles away from the chip surface by doing pulsed injections manually.*
- *Carefully remove syringe from port to avoid backflow.*
- *When necessary, use the Y connector for simultaneous injections in the reference and sample channels.*

3. Fill the reference channel with about 1 ml PBS (no BSA); leave the syringe in the port.
4. Inject 1 ml of thiolated LacO DNA into the sample channel; leave the syringe in the port.
5. Wait until the signal stabilizes in channels A, B and C; about 20 minutes.
6. Remove the sample channel syringe and with a new syringe inject 3 ml of PBS to wash the excess thiolated LacO from the sample channel.
7. Remove both syringes and connect link channels together using the Y connector for the rest of the experiment.
8. Inject 1 ml of MCH; incubate for 30 minutes.
9. Wash the surface using at least 3 ml of PBS.
10. Inject 1 ml of complementary oligomer. Let hybridize until the signal plateaus and stabilizes.
11. Wash the surface using at least 3 ml of PBS + BSA.
12. Ensure you have a stable baseline – up to 5 minutes.
13. Inject 1ml of 5 nM LacI; incubate until the signal plateaus and stabilizes.
14. Sequentially wash with: 3 ml of PBS + BSA, 1 ml of PBS + BSA + SDS, 3 ml PBS + BSA.
15. Repeat steps 14 and 15 using 20 nM, 50 nM, 100 nM and 200 nM LacI.
16. Regenerate the sensor's surface (see steps 19-21) or go to step 18.
17. Press stop and save your experiment (see appendix for data treatment).

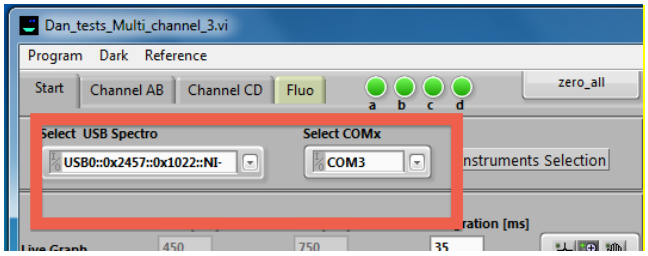
Optional – Regeneration of the sensor.

18. After the last wash sequence, inject the regeneration buffer; incubate 30 seconds.
19. Wash with PBS
20. Repeat step 11 to restart the experiment.

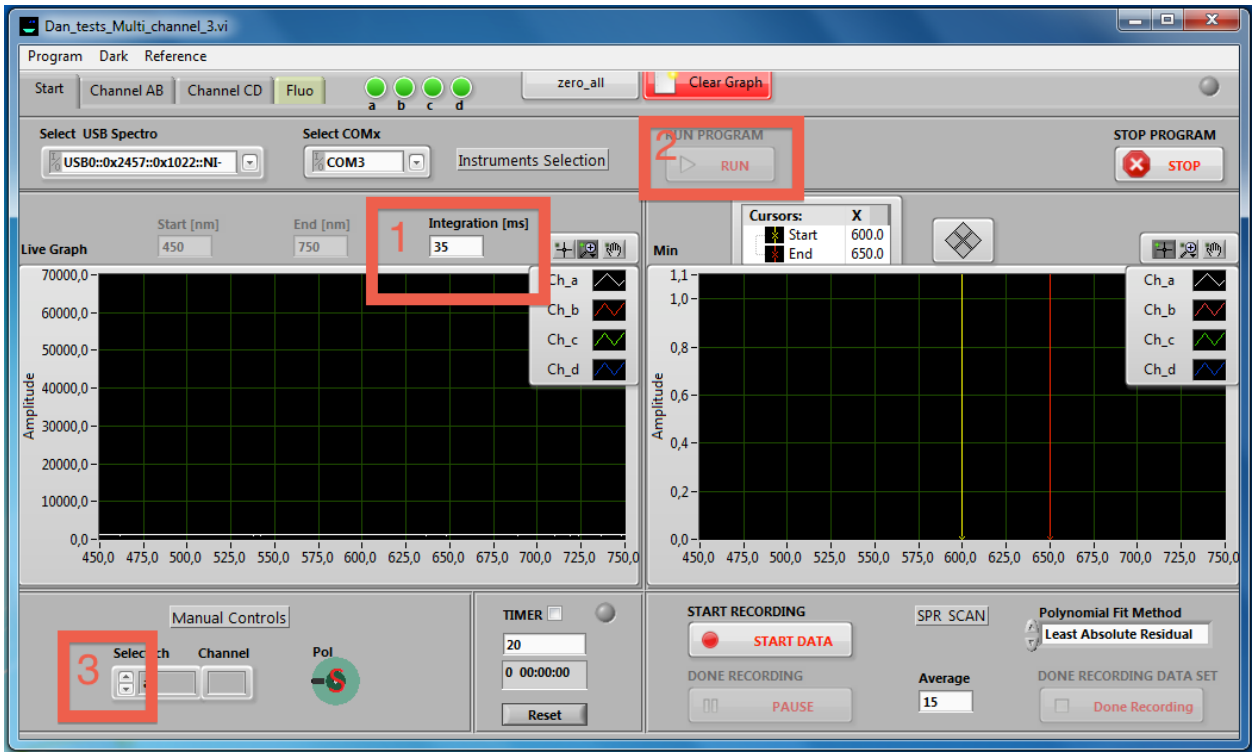
Appendix

P4SPR initiation routine

i. **Select the USB spectro** and **select COMx** using the drop down menu as shown below.



ii. Set the **integration** time 20 ms, click **Run**, and manually select each channel (**Select_ch**) using the arrows to see a signal in the left window.
Note: if the signal is too weak, play with integration time (20 ms to 50 ms).



iii. Ensure the polarizer (**Pol**) is in “S” position; if “P”, click on the letter to change to “S”. In the program menu, click on **Dark** and select **Start Dark**.

iv. Create a folder for your experiment and save the dark files in this folder by click on select this folder (DO NOT click on SAVE).

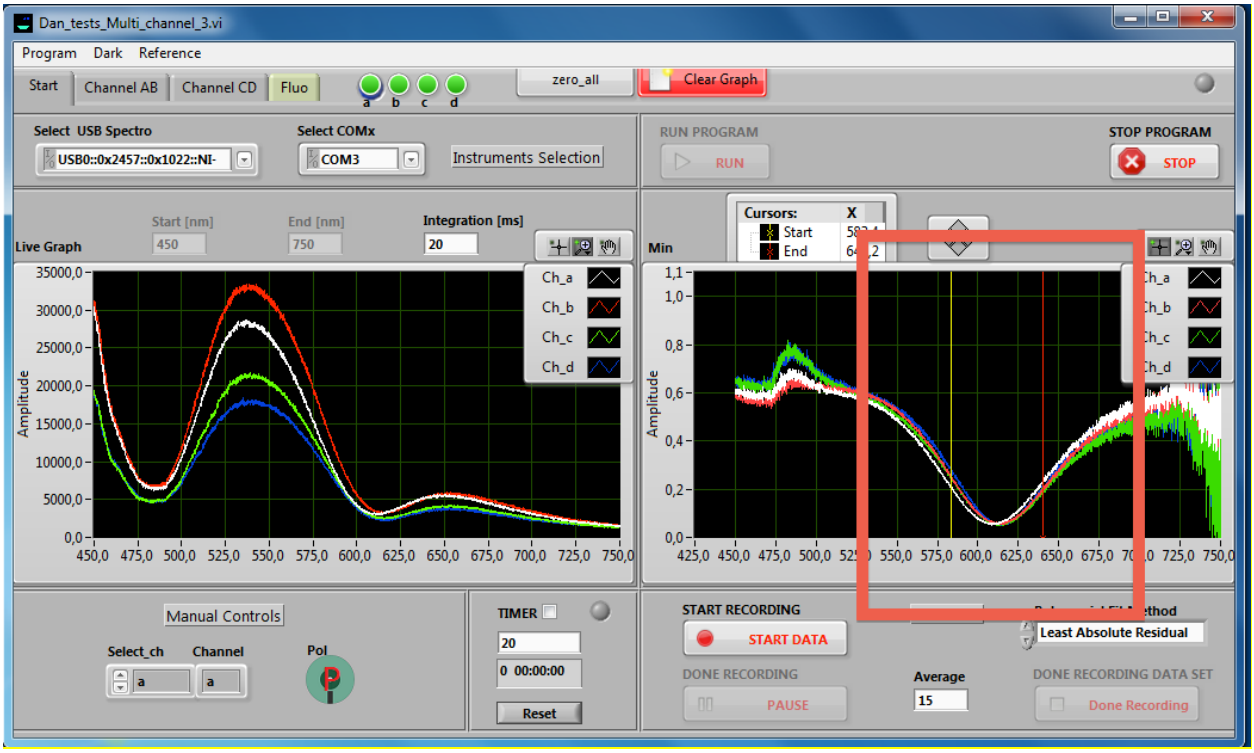
v. Once dark measurements are done, a popup window will appear. Close it.

vi. In the program menu, click on **Reference** and select **Start Reference**.

vii. Save the “Reference” files in the same folder as the “Dark” by click on select this folder (DO NOT click on SAVE).

viii. Once reference measurements are done, a popup window will appear. Close it.
Note: the polarizer should automatically switch to “P” from “S” once the reference measurements are done.

ix. The characteristic SPR absorbance spectrum for each channel (a to d) should appear overlapping in the right side window. On that graph, set the higher and lower tracking boundary (vertical yellow and red lines) about 50 nm apart and ensure the absorption band is in the middle.
Proceed to step x and beyond only if your material is all set for the kinetic experiment.



x. Press on **Start Data** located below the right window. Select the folder in which you saved the "Dark" and "Reference" files and save the kinetic data.

xi. Start an external timer to monitor your experiment.

xii. You can monitor the SPR shift in each individual channel by selecting tabs **Channel AB** and **Channel CD**.

xiii. You can set all channels to zero at all time by clicking on **zero_all**. This adjustment is recorded in your kinetic data file.

xiv. You can also clear the kinetic data from the channel windows by clicking on **Clear Graph**. This adjustment is NOT recorded in your kinetic data file

xv. Once the experiment done, press **Done Recording** and stop your timer.

Retrieve collected data

xvi. Go in the folder where you saved the data and look for four files named **min_a, min_b, min_c,** and **min_d** containing the kinetic data for each channel. Import these data into your data processing software of choice (e.g., excel, origin, matlab, etc.).

xvii. Divide the time of the experiment (in minute) by the total # of data points for a kinetic to get the acquisition frequency.

Details on LacI

Protein sequence:
MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAKTREKVEAAMAELNYIPNRVAQQLAGKQSLLIGVA
TSSLALHAPSQVAAIKSRADQLGASVVVSMVERSGVEACKAAVHNLLAQRVSGLIINYPLDDQDAIAV
EAACTNVPALFLDVSDQTPINSIIFSHEDGTRLGVEHLVALGHQQIALLAGPLSSVSARLRLAGHKYLT
RNQIQPIAEREGDWSAMSGFQQTMQMLNEGIVPTAMLVANDQMAGAMRAITESGLRVGADISVVGY
DDTEDSSCYIPPLTTIKQDFRLLGQTSVDRLLQLSQGQAVKGNQLLPVSLVKRKTTLAPNTQTASPRA
LADSLMQLA RQVSRLESGQHHHHHH

Amino acids: 363

Molecular Weight: 39 kDa

LacI has an affinity for O1 operator sequence in lacO (5'AATTGTGAGCGGATAACAATT3'). It also binds O2 and O3 sequences (respectively 5'AAATTGTAGCGAGTAACAACC3' and 5'GGCAGTGAGCGCAACGCAATT3'). It is a well-known repressor of the lac operon.

LacI purification

Pedigree: Plasmid pBAD-LacI in E.coli TOP10. LacI has a histidine tag at its C-terminus.

1. Stir the ampicillin (100 µg/mL) into LB / agar Broth.
2. Pick a single colony and inoculate 50 mL LB / ampicillin Broth. Grow overnight at 37°C.
3. Put back in suspension 50 ml of the overnight culture in 1 L of LB ampicillin and grow until OD_{600nm} of 0.6.
4. Add 0.2% arabinose (2 g for 1 L) to induce protein expression for 5 hours at 37°C.
5. Centrifuge at 8 K RPM in SLA 3000 for 10 mins.
6. Resuspend the pellet in 40 ml of binding buffer (20 mM Sodium phosphate pH 7, 500 mM Sodium Chloride).
7. Sonicate 2 min at 10 sec intervals with 45 sec delay x 12 cycles.
8. Centrifuge at 4600 g for 20 mins at 4°C.
9. Use the supernatant for LacI purification using FPLC with 5 mL His-Trap™ column (GE HealthCare). Elute with binding buffer supplemented with 500 mM Imidazole.
10. Desalt with Hi-Prep 26/10™ against LBB (LacI Binding Buffer: 10 mM Tris-HCl pH 7.4, 150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA).
11. Store the LacI stock at 10 uM tetramer in LBB supplemented with 30% glycerol. The concentration of LacI tetramer can be measured using the following formula: OD_{280nm} x 10^6 x 22,290 x 4 x optical path length (in cm).
12. Validate purity by SDS-PAGE.

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