qbio
quantitative
biology

SynBio Practicals Guide Part 1

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Introduction

In this practice we will explore the impact of varying regulatory elements on bacterial gene expression using a range of quantitative techniques (plate reader, cytometry) and gene expression systems (living cells and cell-free).

We will perform data collection *in vivo* and *in vitro* to compare the different collection methods and data analysis using different freely available softwares and Python scripts.

You are expected to read and watch the explanatory videos ahead of coming to the class.

You will have to present the results of your analysis during a final oral exam in January 2022.

Schedule

Date	Day		#hours	Time	Subject	Where
23 November	TUE	Day 1	4	13:00-17:00	Microbiology basics and good lab practices	CBS
24 November	WED	Day 2	4	9:00-13:00	DNA extraction and sequencing	CBS
25 November	THU	Day 3	4	13:00-17:00	Fluorescence measurement and sequencing results analysis	CBS
26 November	FRI	Day 4	4	13:00-17:00	Fluorescence measurement and sequencing results analysis	CBS
29 November	MON	Day 5	3	9:00-12:00	Cell free: Introduction and constructs synthesis	CBS
29 November	MON	Day 5	3	14:00-17:00	Cell free: Introduction and constructs synthesis	CBS
30 November	TUE	Day 6	4	13:00-17:00	DNA analysis and quantification	CBS
1st December	WED	Day 7	4	13:00-17:00	Running a cell free experiment	CBS
2 December	THU	Day 8	4	13:00-17:00	Data processing (python)	CBS
3 december	FRI	Day 9	4	13:00-17:00	Data processing and analysis (python)	CBS

Bibliography and resources

You will find here the material to read/watch to carry on the practical. Each day there will be a short explanation of the activities and how we will carry them, but always assuming that you have read this material beforehand.

General stuff and papers

Synthetic Biology One Videos

Evaluation of 244,000 synthetic sequences reveals design principles to optimize translation in Escherichia coli
https://paperpile.com/shared/V9b3Vg/download/5f55e088-ff54-0e2e-b877-198702d11e10

Activities

Day 1
Microbiology basics and good lab practices

General concepts

- Intro to good lab practices
- Labelling
- Pipetting
- Bench organization
- Notebook
- How to prepare media for growing bacteria
- Plating bacteria
- Growing bacteria
- Colony counting
- Colony picking
- Starting ON cultures

Ressources

Videos

Using a micropipette:

Pipette 1

Pipette 2

Colony picking and counting:

Colony picking

Using a centrifuge

Centri 1

Centri 2

Practical part

You will receive erlenmeyers and tubes with bacterial cultures (labelled A, B, C, D, E, F, G, H) and a petri dish with isolated colonies from one of the liquid cultures.

Experiment 1

1. Load a 96 well plate for plate reader (200µL/well) with all samples with undiluted culture, diluted 1/2, diluted 1/10 in LB medium
   Use the template to create your plate map
2. Fill a well with LB medium only and M9 medium only
3. Run the sample on plate reader

Experiment 2

1. Load tubes for flow cytometer with culture diluted 1/200 in focussing fluid, 3mL total
2. Run the sample on the machine

Discuss the results: Which sample is what? What is the main difference between plate and cytometer data?

Experiment 3

1. Observation of the petri dishes under UV light.
2. Pick colonies (3 per person) and start a culture in 5 mL of LB supplemented with Kanamycin 25 ug/mL and grow it at 37C overnight (ON).
3. Pick colonies from negative and positive controls (Negative control (NC), CMN, P7, Ref prom) that will be given to you by the instructors in separate petri dishes and grow them under identical conditions to 2.

Day 2
DNA extraction and sequencing

General concepts

How to do a miniprep

Sequencing procedure

Inoculating clones for follow up experiments

Ressources

Videos

Miniprep:
Miniprep video

Nanodrop:
Nanodrop video

Sequencing basics:
Sequencing video

Readings

A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Birnboim, H. C., and J. Doly. 1979. Nucleic Acids Research 7 (6): 1513–23.

NEB miniprep protocol

Practical part

1. Sterilely keep 500 µL of each overnight culture in an identified 1.5 mL tube. Keep the tubes on ice at 4°C. These will be used to inoculate the deepwell for cytometry analysis.

2. Miniprep:
- centrifuge cultures 3500rpm 8min RT°C.
- Follow NEB miniprep protocol:

a. Pellet 1–5 ml bacterial culture (not to exceed 15 OD units) by centrifugation for 30 seconds. Discard supernatants.
Note: For a standard miniprep to prepare DNA for restriction digestion or PCR, we recommend 1.5 ml of culture, as this is sufficient for most applications. Ensure cultures are not overgrown (12-16 hours is ideal).

b. Resuspend pellet in 200 µl Plasmid Resuspension Buffer (B1) (pink). Vortex or pipet to ensure cells are completely resuspended. There should be no visible clumps.

c. Lyse cells by adding 200 µl Plasmid Lysis Buffer (B2) (blue/green). Invert tube immediately and gently 5–6 times until color changes to dark pink and the solution is clear and viscous. Do not vortex! Incubate for one minute.
Note: Care should be taken not to handle the sample roughly and risk shearing chromosomal DNA, which will co-purify as a contaminant. Avoid incubating longer than one minute to prevent irreversible plasmid denaturation.

d. Neutralize the lysate by adding 400 µl of Plasmid Neutralization Buffer (B3) (yellow). Gently invert the tube until color is uniformly yellow and a precipitate forms. Do not vortex! Incubate for 2 minutes.
Note: Be careful not to shear chromosomal DNA by vortexing or vigorous shaking. Firmly inverting the tube promotes good mixing, important for full neutralization.

e. Clarify the lysate by spinning for 2–5 minutes at 16,000 x g.
Note: Spin time should not be less than 2 minutes. Careful handling of the tube will ensure no debris is transferred and the 2 minute recommended spin can be successfully employed to save valuable time. For culture volumes > 1 ml, we recommend a 5 minute spin to ensure efficient RNA removal by RNase A. Also, longer spin times will result in a more compact pellet that lowers the risk of clogging the column.


To save time, spin for two minutes only.

f. Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.


To save time, spin for 30 seconds, instead of 1 minute.

g. Re-insert column in the collection tube and add 200 µl of Plasmid Wash Buffer 1. Plasmid Wash Buffer 1 removes RNA, protein and endotoxin. (Add a 5 minute incubation step before centrifugation if the DNA will be used in transfection.) Centrifuge for 1 minute. Discarding the flow-through is optional.
Note: The collection tube is designed to hold 800 µl of flow-through fluid and still allow the tip of the column to be safely above the top of the liquid. Empty the tube whenever necessary to ensure the column tip and flow-through do not make contact.


To save time, spin for 30 seconds, instead of 1 minute.

h. Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.

i. Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column has not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute before inserting it into the clean microfuge tube.

j. Add ≥ 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.
Note: Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Delivery of the Monarch DNA Elution Buffer should be made directly to the center of the column to ensure the matrix is completely covered for maximal efficiency of elution. Additionally, yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated as a result of dilution. For larger plasmids (≥ 10 kb), heating the DNA Elution Buffer to 50°C prior to eluting and extending the incubation time after buffer addition to 5 minutes can improve yield.

- Measure concentration with nanodrop (Nanodrop video)

3. Send for Sanger sequencing 5µL primer P40 (Sp5 Rev) at 10µM + 5µL DNA
Then 5µL primer P102 (Fw in pSB4K5) at 10µM + 5µL DNA (48 tubes)
Do not sequence P7 and Ref prom
Sequencing video
Keep the DNA at -20°C until the PCR manipulation for cell free

4. Split in two groups (group #1 and #2)

5. **Group #1** sterilely inoculate clones and controls in triplicates in 96 deep-well plate:
500µL M9 0.4% glycerol Kana25/ well
+ 5 µL culture kept before
Apply breathable adhesive seal
→ 16h at 37°C in Kuhner incubator (kept at 4°C until 7 pm, then an instructor will put it for overnight growth at 37°C)

Keep the 1.5mL tube at 4°C until tomorrow to inoculate the plate for plate reader then the deep well for group #1

Plate distribution

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	*CMN*	*CMN*	*CMN*	*RefP*	*RefP*	*RefP*	*P7*	*P7*	*P7*	*NC*	*NC*	*NC*
B	*1*	*1*	*1*	*2*	*2*	*2*	*3*	*3*	*3*	*4*	*4*	*4*
C	*5*	*5*	*5*	*6*	*6*	*6*	*7*	*7*	*7*	*8*	*8*	*8*
D	*9*	*9*	*9*	*10*	*10*	*10*	*11*	*11*	*11*	*12*	*12*	*12*
E	*13*	*13*	*13*	*14*	*14*	*14*	*15*	*15*	*15*	*16*	*16*	*16*
F	*17*	*17*	*17*	*18*	*18*	*18*	*19*	*19*	*19*	*20*	*20*	*20*
G	*21*	*21*	*21*	*22*	*22*	*22*	*23*	*23*	*23*	*24*	*24*	*24*
H	M9 only	M9 only	M9 only									

Optionally: pick 9 colonies from 244k library plate to fill the plate and see what we get!

Day 3

Fluorescence measurement and sequencing results analysis #1

General concepts

-Bulk measurements vs. single cell

- Kinetic vs end-point measurement

-Advanced plate reader and flow cytometer functioning and manipulation

Ressources:

Plate reader
Link to Plate reader protocol

Cytometer:
Flow cytometry 1
Attune flow cytometer

Software
Benchling : open an account on benchling at https://www.benchling.com/
Send your email to elsa.fristot@cbs.cnrs.fr

Practical part (students are split in two groups and exchange tasks between day 3 and 4)

Group #1:

1. Preparation of sample plate for plate-reader **end point** measurement (non sterile 96 wells plate)
    - ☐ Sterilely fill a black body 96 well plate (flat transparent bottom) with 100 µL M9 defined culture medium
    - ☐ Sterilely add 100 µL from overnight deep-well culture plate

2. Preparation of sample plate for plate-reader **kinetic** measurement (sterile 96 wells plate)
    - ☐ Sterilely fill a black body 96 well plate (flat transparent bottom) with 198 uL M9 defined culture medium supplemented with 0.4% Glycerol + Kan 25 ng/µL
    - ☐ Sterilely seed with 2 µL from overnight deep-well culture plate (for kinetic measurement)
    - ☐ Seal the plate with transparent adhesive cover
3. Run the plates in plate reader with following settings:

- ☐ 37°C heating
- ☐ Orbital shaking
- ☐ Abs 600nm (equivalent Optical Density)
- ☐ GFP ex/em 485/528; use two gains gain 70 and 100
- ☐ Measure every 10 min for 8h (kinetic measurements)

Link to Plate reader protocol

Group #2:

1. Start the flow cytometer
2. Fill a transparent 96 well plate with 199 µL focusing fluid
3. Sterilely add 1 µL from overnight deep-well culture plate
4. Run the flow cytometer on the whole plate (~1h) with the following setting:
    - ☐ Blue laser (488 nm)
    - ☐ 510/10 BP filter (BL1 for GFP)

Link to Flow cytometer Protocol

5. Prepare deep-well plate for next day experiments using tubes stored in the fridge, use same protocol as the day before

Group #1 & 2
Benchling with Elsa
Open an account and send you user email to elsa.fristot@cbs.cnrs.fr

Day 4

Fluorescence measurement and sequencing results analysis #2

Group #1 : do flow cytometry as in day 3
Group #2 : do plate reader as in day 3

Group #1 & 2
Benchling with Elsa

Group #1 and 2:
Finish sequencing analysis, recover data from the day before, start processing if possible