

Lab book Nessim Louafi

MARDI 23/11/2021

Day 1 : Setup of the cultures and preliminary observations

1. End point plate reader

96 plate for plate reader:

Each group filled 3 columns with 3 different dilution and fills as much rows as different colonies (see Well1 for the layout)

300 μ L prepared for 200 μ L deposited in each wells as follow

Dilutions:

- 1/2: 150 μ L of bacteria culture + 150 μ L of LB medium.
- 1/10: 30 μ L of bacteria culture + 270 μ L of LB medium.

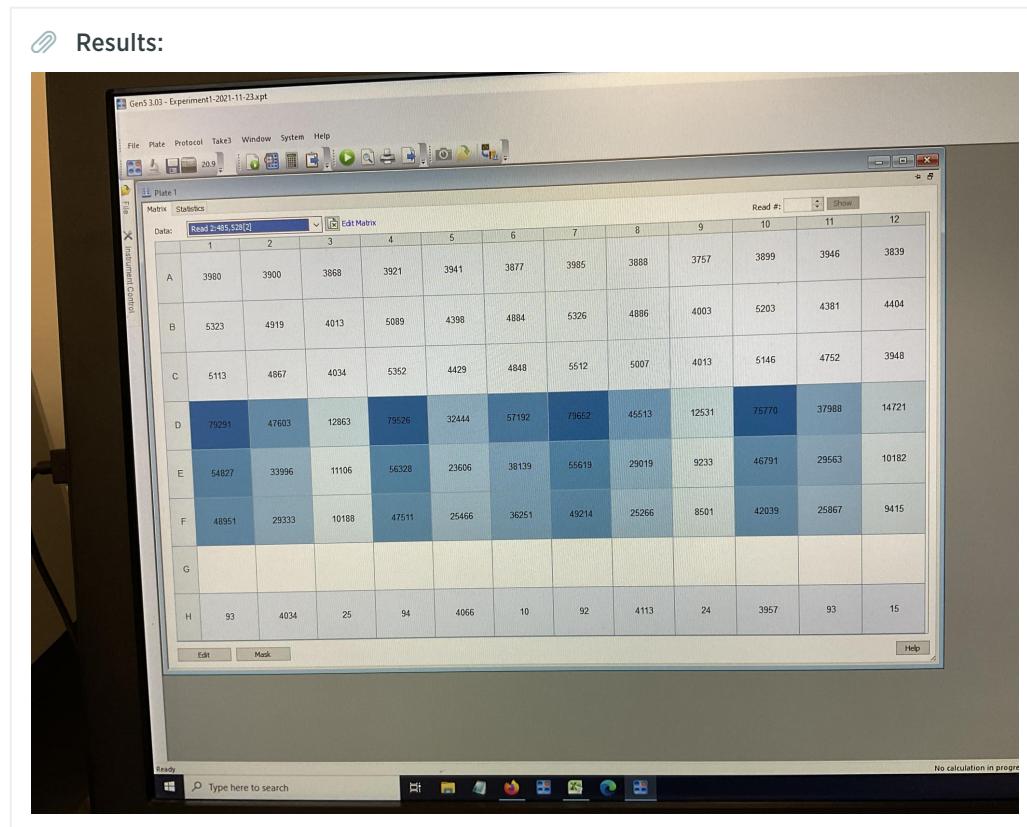
Plan of the plate:

C1 : LB only

C2: M9 only

(this will be used to determine background noise)

Well1												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Col A 1:1	Col A 1:2	Col A 1:10									
B	Col B 1:1	Col B 1:10	Col B 1:2									
C	Col C 1:1	Col C 1:2	Col C 1:10									
D	Col D 1:1	Col D 1:2	Col D 1:10									
E	Col E 1:1	Col E 1:2	Col E 1:10									
F	Col F 1:1	Col F 1:2	Col F 1:10									
G	X	X	X									
H	C1	C2	X									



See code for further analysis

2. Flow cytometer of colonies

For each of the 6 cultures (A to F) :

Sample preparation : 1/200 of each cultures in 3mL of focusing fluid:

- 15µL of bacteria culture + 2985 µL of focusing fluid

Settings for cytometer: Blue laser (488nm) , 510/10 BP filter (BL1 for GFP)

Results (visual) :

- A -> NC
- B -> CMN (promoter CMN no GFP)
- C -> RefP (GFP + reference promoter : positive control)
- D -> P7 (also reference promoter but higher fluorescence expected)
- E -> C+D mixture
- F -> Mixture of all others

3. Pick colonies and start cultures

Observation of petri dishes containing all the colonies under UV light (to see GFP and make sure the experiment works)

- 3 colonies picked : 19,20,21
- Start a culture in 5mL of LB supplemented with 25µg/mL of Kanamycin:
 - dilution 1/2000 (50mg/mL) 100µL in 200mL
- Control picked C1 and LB alone
- Grow the colonies overnight at 37°C

Day2: Preparation of the samples for sequencing

Start with 500µL of overnight culture (day before), keep at 4°C.

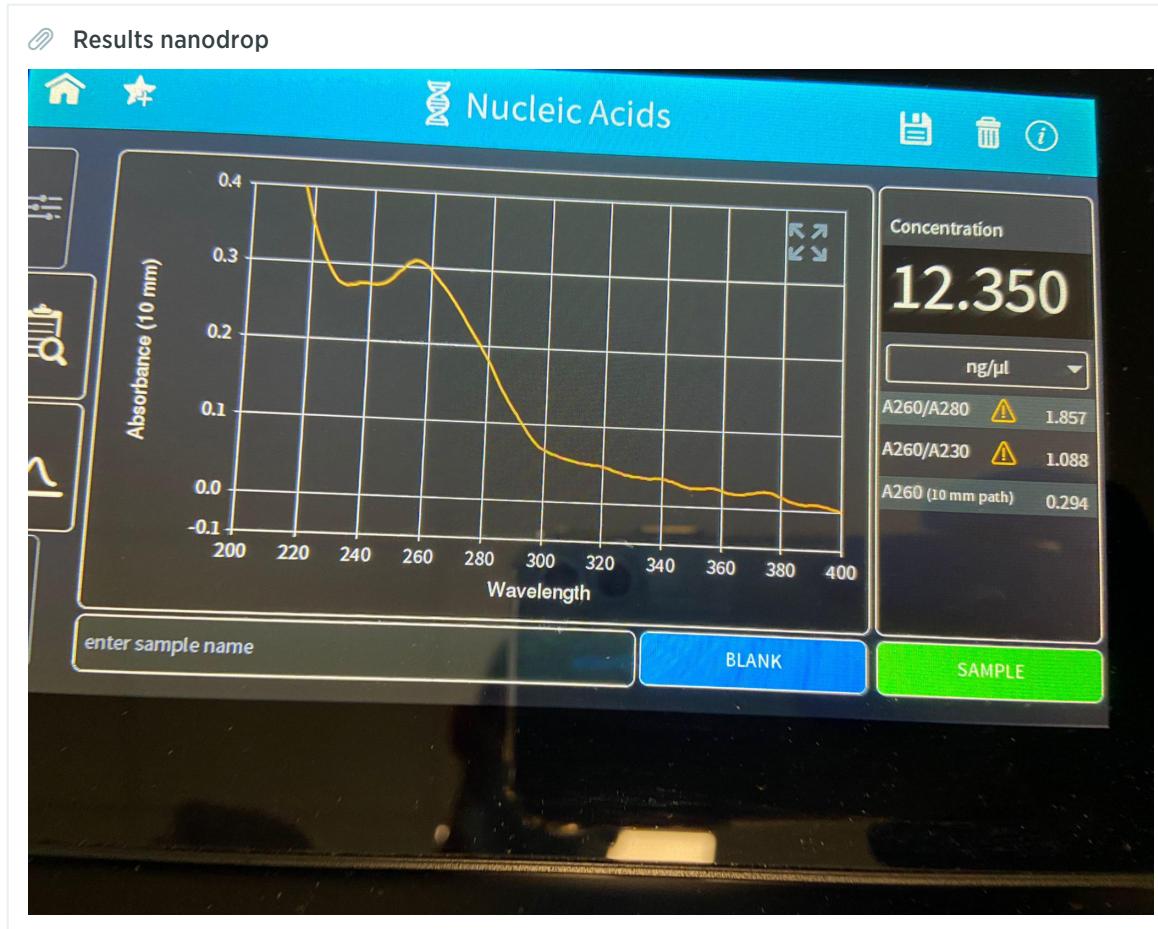
1. Miniprep

From NEB miniprep protocol:

- Centrifuge culture 3500 rpm for 8min at room temperature
- Resuspend pelet : Keep pelet, resuspend in 200µL of plasmid resuspension buffer B1. Vortex or pipete to completely resuspend (no clumps).
- Lyse cells : 200µL of plasmid lysis buffer B2. Invert tube 5-6 time **gently** until color switch to dark pink incubate for 1 min (**don't vortex**).
- Neutralize the lysate : 400µL of plasmid neutralization buffer B3. Invert **gently** until color is uniformly yellow + presence of precipitate, incubate for 2 min (**don't vortex**).
- Clarify the lysate: spin for 2-5 min at 16,000g
- Keep supernatant : transfer it to the spin column , insert it in a tube and centrifuge for 1 min .
- Wash 1: discard the flow-through: insert the column in the collection tube and add 200µL of wash buffer 1 (remove RNA protein and endotoxin). Centrifuge for 1 min, discard the flow-through.
- Wash 2: add 400µL of plasmid wash buffer 2 and centrifuge for 1 minute.
- Transfer to elution : transfer column in a 1.5 mL microfuge tube (**carefull** not to touch flow-through with column when transferring, if so respin for 1 minute before transferring) .
- Elution: add >30µL DNA elution buffer to the center of the column, incubate for 1 min and spin for 1 minute to elute.

2. Measure concentration on nanodrop (test the purity and verify the miniprep worked)

Measure concentration of purified DNA on nanodrop:



3. Send for sequencing

- Combine all miniprep DNA

Make 2 tube (one for each primer) as follow:

2 different primers : P40 and P102

- 5µL of primer at 10µM + 5µL DNA
- Keep the DNA at -20°C**

4. Inoculate clones

Sterily inoculate clones and controls in triplicates:

Content: 500µL of M9 medium with 0.9% glycerol Kana25 + 5µL of culture /well as described below

Well2

	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	M9	M9									

Apply **breathable seal** and incubate for 16h at 37° C

JEUDI 25/11/2021

Day 3: Fluorescence measurement and analysis of the sequencing results

1.End point plate reader

Same layout as 24/11/2021, 5X dilution in 200µL M9 media :

160 µL M9+ 40µL deep-well culture from day before

2.Kinetic plate reader

Same layout as 24/11/2021 (take a transparent bottom 96 well plate for measurements):

Sample preparation : 198 µL M9 with 0.4% Glycerol + Kan 25ng/µL + 2µL deep-well culture from day before

Run the plate in plate reader with:

- 37°C
- Orbital shaking
- Abs 600nm (OD)
- GFP ex/em 485/528 (gain 70 and 100)
- Measure every 10 min for 8h

3. Analysis of the results of sequencing

Analysis on benchling by sequence alignments

Table1

	A	B	C	D	E	F
1	Barcode	Colony number	Primer number	Observation	Promoter	Observation
2	ETJ218	21	40	Sequencing of GFP (not full) + promoter match with D03 prom J54200 + insertion at position 534	D03-promJ54200-in-pSB4K5-fromCMN	Creation of a stop site at position 14 on GFP (might have an effect on fluo production)
3	ETJ219	19	40	Match with promoter K2832128 + sp0	A06-promK2832128-in-pSB4K5-fromCMN	Normal promoter no mutations
4	ETJ220	20	40	Sequencing did not work (sequence too short)		
5	ETJ221	20	102	Sequencing only GFP sequence (not entirely) and no promoter region.		
6	ETJ222	19	102	No match with promoter because sequencing was not displaying promoter region only GFP + beginning of promoter		
7	ETJ223	21	102	Sequencing fine but no match for promoter, only the GFP sequence was sequenced so no match for promoter		

VENDREDI 26/11/2021

Day 4: Single cell fluorescence measurement on flow cytometer

1.Flow cytometer on culture plate (5days)

Same layout as well 2 (24/11/2021)

199µL focusing fluid + 1µL of deep-well culture

Settings for cytometer: Blue laser (488nm) , 510/10 BP filter (BL1 for GFP)

2.Prepare deep well for next day experiment

Same protocol as 24/11/2021 , layout : well 2

LUNDI 29/11/2021

Day 5 : Constructs amplification for PCR experiments

PCR for cell free experiment

- **Mastermix:**

8 reactions, 160µL final volume :

- 5X superfi buffer: 32µL
- 10mM dNTP: 3.2µL
- DNA pol : 3.2 µL
- Primer (10µM) : 8µL
- Primer (10µM): 8µL
- Water : 101.6µL

For each reactions : 19.5 µL of master mix + 0.5µL of DNA

- **Cycle:**

- 98° C for 30'
- 98° C for 10'
- 60°C for 10' annealing
- 72°C for 30sec for elongation
- 72°C for 5 min for final elongation
- 4°C at the end to cool off

PCR product will be 1123 bp so 30sec elongation time (30seconds per kb) (primers [33](#) [34](#))

For which colonies were picked see well 2 (24/11/2021)

MARDI 30/11/2021

Day 6 : Quantification of PCR and quality evaluation of DNA amplified

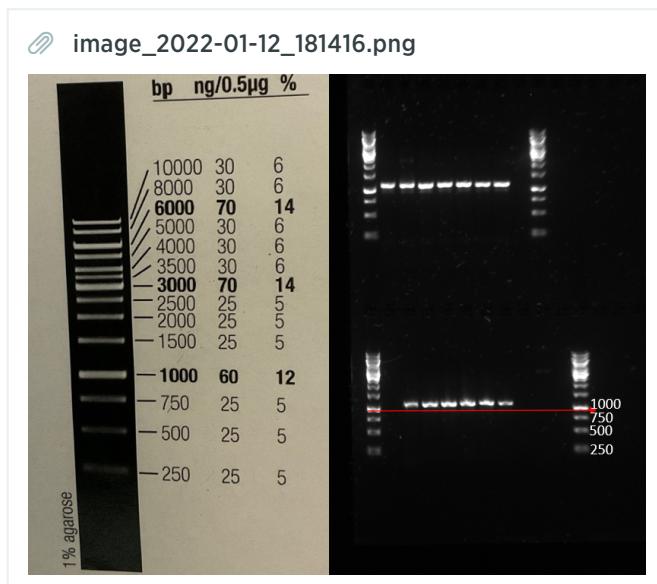
1.Prepare agarose gel for PCR verification

- 0.8% (m/m) agarose in 100 mL (0.8 g of solid agarose)
- Melt agarose
- dilute uin TAE 0.5X (~200mL)
- Add 4µL cybersafe (intercalling agent) to dye the bands (**!TOXIC**)
- Prepare DNA : 1µL DNA (each colonies) +4µL H₂O + 1µL of 6X loading dye
- Load gel with 6µL of the prepared solution

2.Run gel

40 minutes at 100V

Image on Gel Imaging System

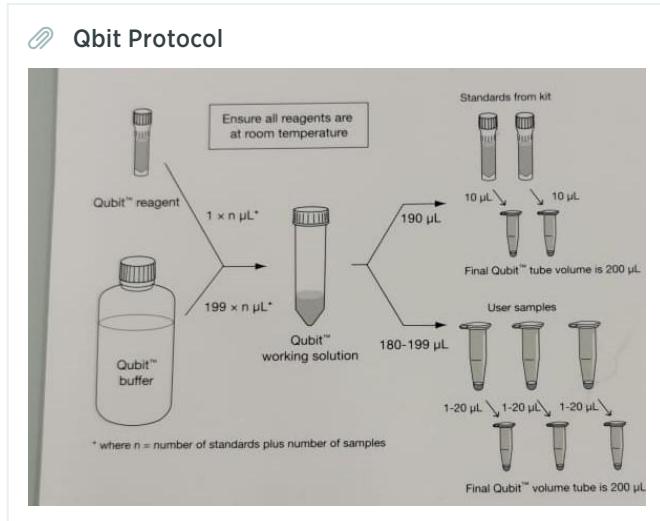


3.Measure PCR product by Qubit

Following protocol provided by the manufacturer (see below) :

- Sample preparation :

- Preparation of the master mix :
 - 3.2 mL of Qubit buffer
 - 16 µL of Qubit reagent
- Preparation of the standard solutions (0 and 100 ng/µL), for each :
 - 198µL of the master mix solution
 - 10µL of the standard solution
- Preparation of the sample tubes, for each:
 - 198µL of the master mix solution
 - 2µL of DNA (from the PCR see day before)
- Vortex samples and wait for 5 minutes
- Quantify the samples using the Qbit fluorometer (using the standard for calibration)



2.Dilute DNA fragments for cell-free

Dilution as follow :

DNA fragment dilution for cell free							
	A	B	C	D	E	F	G
1	Sample name	Size (bp)	[DNA] ng/ μ L	[DNA] nM	H ₂ O to add (μ L)		
2	p7	1123	77,3	111,38	20,9		$C_f = (C_i * V_i) / V_f$
3	1	1123	82,7	119,16	23,5		$V_f = V_i + x\mu L$
4	4	1123	83,4	120,17	23,9		$x = ((C_i * V_i) / C_f) - V_i$
5	5	1123	134	193,08	48,6		$x = V_i * ((C_i / C_f) - 1)$
6	6	1123	100	144,09	32,0		$x = H_2O \text{ to add to have } C_f \text{ nM } (\mu L)$
7	10	1123	95,4	137,46	29,7		
8	14	1123	105	151,30	34,4		
9	16	1123	126	181,55	44,7		
10	18	1123	124	178,67	43,7		
11	19	1123	92,8	133,72	28,5		
12	20	1123	92,4	133,14	28,3		
13	22	1123	97,2	140,06	30,6		

MERCREDI 01/12/2021

Day 6 : Cell free experiment

1. Cell free master mix preparation

- Prepare for n + 10% reaction here 90 reactions prepared
- Master mix volume per reaction : 22 μ L (with DNA)
- Composition of the master mix for 1 reaction :
 - 9.17 μ L of cell-free buffer (amino acids, co-factors, energy regeneration system, crowding agent, salt)
 - 7.33 of bacterial extract (see protocol below)
 - 4.4 μ L of DNA
 - 1.1 μ L of γ S (protect linear DNA)
- For 90 reactions : 660 μ L of extract, 825 μ L of cell free buffer, 99 μ L of gamma S. Add the DNA after directly inside the well

Layout :

Cell-free plate layout																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12											
C		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12											
D		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12											
E			B1	B2	C1	C3	C4	C8	C9	C10	D1	D7	Inco 20											
F			B1	B2	C1	C3	C4	C8	C9	C10	D1	D7	Inco 20											
G			B1	B2	C1	C3	C4	C8	C9	C10	D1	D7	Inco 20											
H	P7 linear	P7 plasmid	H20																					
I	P7 linear	P7 plasmid	H20																					
J	P7 linear	P7 plasmid	H20																					
K																								
L																								
M																								
N																								
O																								
P																								

Parameters of the plate reader for cell free:

- Pre-heat to 37 ° C
- Run kinetics experiment with:
 - Excitation: 485
 - Emission: 528
 - gain : 70/100
 - run for 8 hours