Date : 23rd August ‘18

PCR of mCherry, pCL001 (from Jake 22nd August ‘18) **using Q5 HF polymerase:**
DNA concentration before PCR, **110 ng/µl.**
Dilution of the plasmid to a concentration of 3ng/µl.

1) Set up 50µl reactions in following way :

Experiment

Component	Volume (in ul)
Q5 HF 2x master mix	25
10 uM forward primer	2.5
10 uM reverse primer	2.5
Template DNA	1
Water nuclease free	Upto 50

Negative control

Component	Volume (in ul)
Q5 HF 2x master mix	25
10 uM forward primer	2.5
10 uM reverse primer	2.5
Water nuclease free	Upto 50

Note : Thaw the master mix gently and may be quick spin of 5 seconds after thaw is recommended.

2) Thermocycling conditions for PCR

Step	Temperature	Time
Initial Denaturation	98℃	1 minute
30 cycles	98℃	10 seconds
	65℃	30 seconds
	72℃	40 sec
Final Extensions	72℃	5 minutes
Hold	4℃	∞

PCR of plasmid duet, pCL002 (from Jake 22nd August '18) **using Q5 HF polymerase:**
DNA concentration before PCR : **250 ng/µl.**
Dilution of the plasmid to a concentration of 1ng/µl.

1) Set up 50 ul reactions in following way :

Experiment

Component	Volume (in ul)
Q5 HF 2x master mix	25
10 uM forward primer	2.5
10 uM reverse primer	2.5
Template DNA	1
Water nuclease free	Upto 50

Negative control

Component	Volume (in ul)

Q5 HF 2x master mix	25
10 uM forward primer	2.5
10 uM reverse primer	2.5
Water nuclease free	Upto 50

Note : Thaw the master mix gently and may be quick spin of 5 seconds after thaw is recommended.

2) Thermocycling conditions for PCR

Step	Temperature	Time
Initial Denaturation	98℃	30 seconds
30 cycles	98℃	10 seconds
	61℃	30 seconds
	72℃	2 minutes
Final Extensions	72℃	5 minutes
Hold	4℃	∞

Preparation of 1% agarose gel (Protocol 3)

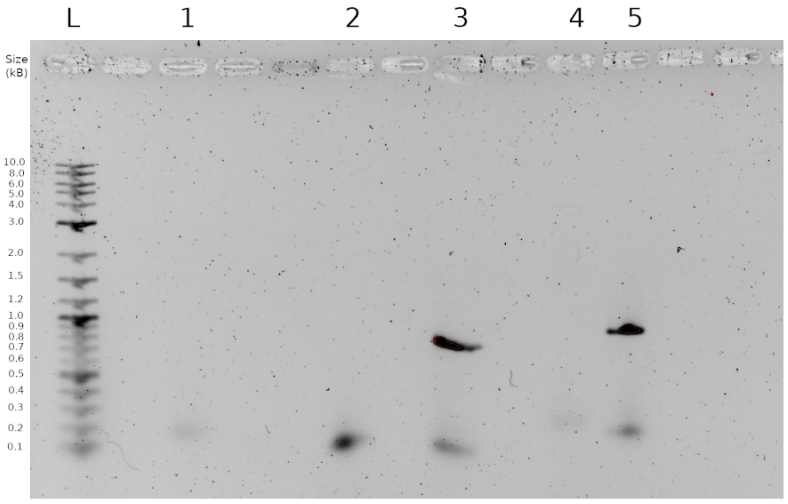
1) Weight 1g of agarose and put the powder in the erlenmeyer.
2) Add 100 ml of TAE buffer (1X) to it.
3) Microwave the erlenmeyer for 2 minutes.
4) Let the gel cool down and add 2ul of SYBR safe to it.
5) Pour the liquid in the casting tray. Place the comb.
6) Wait until complete polymerization.

Verification of PCR products

1) Load 1ul of DNA samples + 1 ul of loading DYE (6X) + 4 ul of dH2O.
Load 1ul of ladder + 1ul of loading Dye and 4 ul of water.

2) Run the gel for 90 minutes at 100 volt,
3) Visualise the gel.

Results



L : ladder
1 : pDuet control
2 : pDuet
3 : mCherry
4 : mCherry control
5 : mCherry

The amplification of mCherry is successful (theoretical mass : 770 bp). However there bands present specific to dimers of incomplete polymerization reactions which might interfere during the cold fusion reaction. The band in well 3 is diffused because it was loaded before the last samples.
On the contrary, the amplification of pDuet (theoretical mass : 3471 bp) failed.

Troubleshooting PCR for pDuet

1) Preparation of new dilution of pDuet with a concentration of 5ng/ul.
2) Set a gradient PCR of 10ul *8 each with a variation of annealing temperature from 58℃ to 65℃ and a negative control.

Experiment

Component	Volume (in ul)
Q5 HF 2x master mix	40
10 uM forward primer	4

10 uM reverse primer	4
Template DNA	1,6
Water nuclease free	Upto 80

Negative control

Component	Volume (in ul)
Q5 HF 2x master mix	5
10 uM forward primer	0,5
10 uM reverse primer	0,5
Water nuclease free	Upto 10

3) Thermocycling conditions for gradient PCR

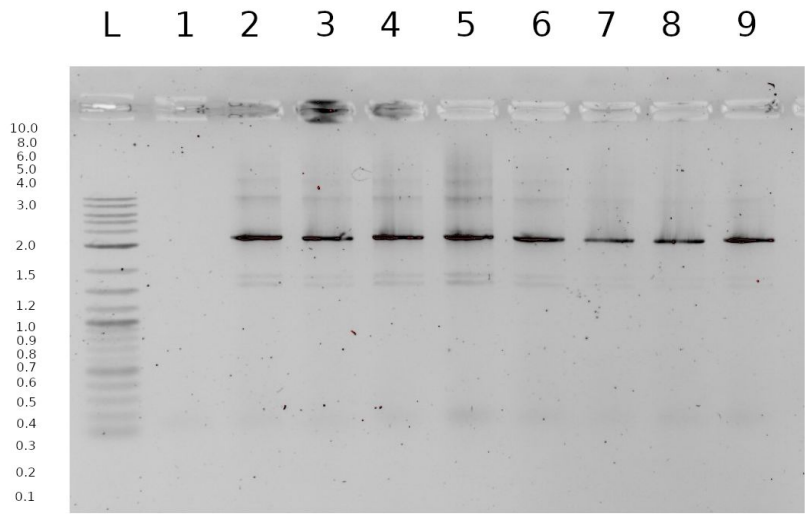
Step	Temperature	Time
Initial Denaturation	98℃	30 seconds
30 cycles	98℃	10 seconds
	58 to 65℃	30 seconds
	72℃	2 minutes
Final Extensions	72℃	5 minutes
Hold	4℃	∞

Verification of PCR products

1) Prepare the 1% agarose gel
2) Load the sample (1µl DNA, 1µl of loading dye, 4µl of water)
3) Run the gel 90 min at 100V

4) Visualise the gel

Result :



1 : pDuet control
2-9 : pDuet (58 to 65°C)

PCR of pDuet worked at all annealing temperatures (between 58 to 65℃). The dilution of the template might be the issue of the first PCR.

DpnI digestion

Set up of the reaction :

Component	Volume (in ul)
DpnI (20,000 units/ml)	1 ul
pDuet PCR product	80µl

All pDuet PCR products were pooled together and digested for 37°C for 4h.

PCR cleanup for pDuet and mCherry PCR products

The cleanup was realized by following the protocol provided by the company (*A&A biotech clean up*)

1) Mix DNA samples with 5 volumes of GI binding solution. Mix by inverting the tubes or vortexing. Apply the samples onto the wells of the purification column.
2) Centrifuge for 1 min at 2000 x g. Discard the filtrates.
3) Add 600 µl of A1 wash solution.
4) Centrifuge for 2 min at 2000 x g.Discard the filtrates.
5) Add 300 µl of A1 wash solution.
6) Centrifuge for 10 min at 2000 x g. Discard the filtrates.
7) Wait few minutes for ethanol to evaporate completely.
8) Place the purification column on the elution tube.
9) Add 50 µl sterile water and incubate for 3 min at room temp.
10) Centrifuge for 3 min at 2000 x g.

Result : Nanodrop concentration of pDuet **210ng/ul** and mCherry **93ng/ul**.

Cold Fusion Assembly (System Bioscience)

1) Set up 10ul of cold fusion reaction in 1.5 ml reaction tube by mixing the following reagents.

Note : Molar ratio of insert:vector, 2:1.

Experiment (Cloning Reaction)

Total reaction volume : 10ul

Component	Volume
Linearised destination vector (10-100 ng/ul)	0.5ul
PCR insert (20 -200 ng/ul)	0.5ul
dH2O	7ul
5x master mix	2ul

Positive control (provided in kit)

Total reaction volume : 10ul

Component	Volume
Linearised vector (positive control)	1ul
PCR insert (positive control)	1ul

dH2O	6ul
5x master mix	2ul

Negative control

Total reaction volume : 10ul

Component	Volume
Linearised vector (10-100 ng/ul)	1ul
dH2O	7ul
5x master mix	2ul

2) Incubation 5 min at RT
3) Incubation 10 min on ice

Transformation

1) Add 50ul of competent cells provided by the kit to the cloning mixture
2) Incubate on ice for 20 minutes
3) Heat shock at 42˚C for 50 seconds
4) Transfer on ice for 2 minutes
5) Add 250ul S.O.C medium or LB broth
6) Incubate at 37˚C for 1h
7) Take 100 ul culture spread on pre-warmed (37˚C) Spectinomycin plate (or Ampicillin for the positive control)
8) Incubate the plate at 37˚C overnight

Troubleshooting PCR of mCherry PCR

1) Preparation of dilution of the plasmid containing mCherry at 7ng/ul.
2) Set a gradient PCR of 10ul *8 each with a variation of annealing temperature from 60℃ to 68℃ and two negative control.

Experiment

Component	Volume (in ul)
Q5 HF 2x master mix	40
10 uM forward	4

primer	
10 uM reverse primer	4
Template DNA	1,6
Water nuclease free	Upto 80

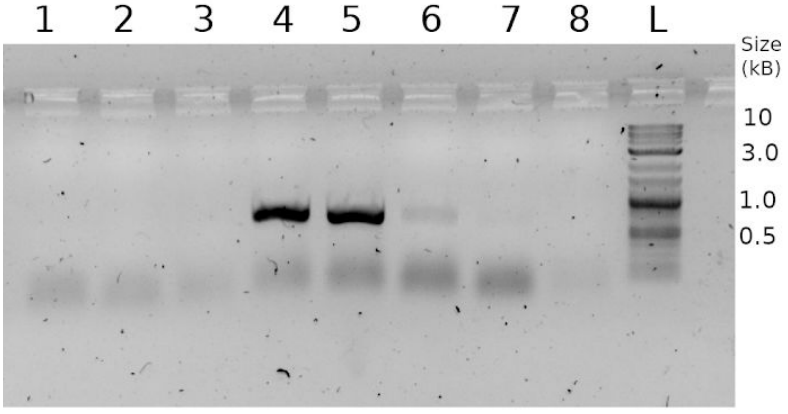
Negative control

Component	Volume (in ul)
Q5 HF 2x master mix	5
10 uM forward primer	0,5
10 uM reverse primer	0,5
Water nuclease free	Upto 10

Date : 25th August ‘18

Result of transformation: No colonies on any of the plate. Either transformation or cold fusion failed.

Confirmation of PCR



1-8 : mCherry (60-68°C)
L : Ladder

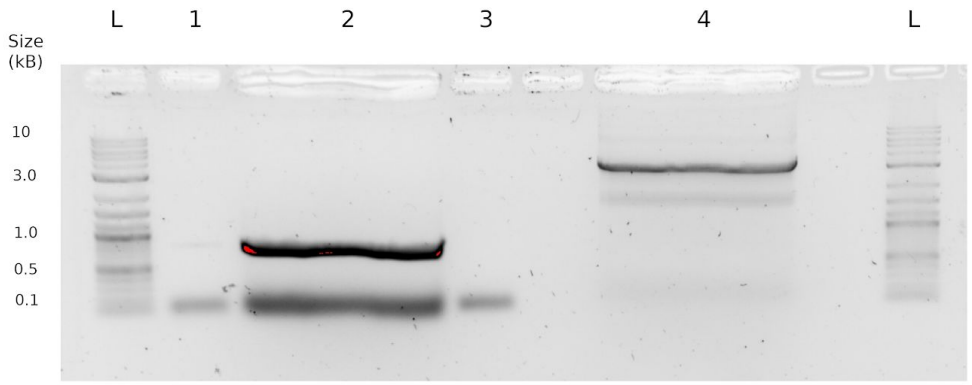
mCherry PCR is successful for mCherry at 63.1℃ and 64.9℃ annealing temperature. Dimers are still present. A gel separation and purification of the correct size mCherry PCR might be a solution.

Date : 26th August ‘18

PCR of mCherry

Set a PCR of 45ul *2 each with a annealing temperature at 63.5℃ and 64.9℃ and two negative control of 5ul for each PCR, as previously.

Confirmation of PCR



L : ladder
1: mCherry negative control
2 : mCherry
3 : pDuet negative control
4 : pDuet

Gel extraction of PCR product for mCherry and plasmid Duet (Protocol for gel extraction)

Result : Gel extraction is unsuccessful and percentage of DNA is quite low.

Date : 27th August ‘18

PCR of mCherry

Gel extraction : Promega kit

Result : Gel extraction is unsuccessful and peak at nanodrop is very weird.

Date : 28th August

PCR mCherry

Gel extraction : Promega kit

Cold Fusion Assembly (System Bioscience)

1)The reaction was set up with the same reagents. (Molar ratio of insert:vector, 2:1).
2) Incubation 5 min at RT
3) Incubation 10 min on ice

Transformation

4) Add 50ul of competent cells provided by the kit to the cloning mixture
5) Incubate on ice for 20 minutes
6) Heat shock at 42˚C for 30 seconds
7) Transfer on ice for 2 minutes
8) Add 1ml of LB broth
9) Incubate at 37˚C for 1h
10) Centrifuge the tube 5 min at 6000 rpm, remove the supernatant and resuspend the pellet in 100 µl
11) Spread the culture on pre-warmed (37˚C) Spectinomycin plate (or Ampicillin for the positive control)
12) Incubate the plate at 37˚C overnight

Date : 29th August

Result of transformation: 15 colonies on the experiment plate, 7 on the positive control plate and 0 on the negative control plate.

Overnight culture
1) Prepare 8 falcon tubes by adding 5 ml of LB + 50µg/ml of Spectinomycin
2) Pick 8 isolated colonies and inoculate the tubes
3) Incubate overnight at 37°C

Date : 30th August

Miniprep of Cold Fusion products (A&A Biotech)

The miniprep was realized by following the protocol provided by the company (*A&A Biotech miniprep*)

1) Centrifuge the overnight bacterial cultures
2) Remove the supernatants.
3) Suspend the bacterial pellets in 200 µl of L1 cell suspension solution.
4) Add 200 µl of L2 lysis solution and gently mix. Incubate for 3 min at room temperature.
5) Add 400 µl of GL3 neutralizing solution and gently mix until the disappearance of raspberry colour of the lysates.
6) Centrifuge the lisates for 10 min at 10 00 rpm
7) Transfer the supernatants onto the minicolumns.
8) Centrifuge for 1 min at 10 000 rpm
9) Discard the filtrates and add 500 µl of W first wash solution.
10) Centrifuge for 1 min at 10 000 rpm
11) Discard the filtrates and add 600 µl of A1 second wash solution.
12) Centrifuge for 2 min at 10 000 rpm
13) Transfer the columns to new eppendorf tubes.
14) Add 60 µl of sterile water (not included) directly onto the minicolumns resin.
15) Incubate for 3 min at room temperature.
16) Centrifuge for 1 min at 10 000 rpm.

Glycerol stock of overnight culture

1) Add 500 µl of cell to 500 µl of 30% glycerol in cryotubes
2) Store at -80°C

BbsI digestion

For 10* 10 µl reactions :

Component	Volume (in ul)
BbsI HF	0.5
Cutsmart buffer (10X)	10
Water nuclease free	Upto 80

1) Split the master mix in 10 PCR tubes
2) For 8 tubes, add 1µl of the miniprep cold fusion plasmids.

3) Add 1 ul of water in the last two tubes (control)
4) Incubate 1h at 37°C

Gel confirmation

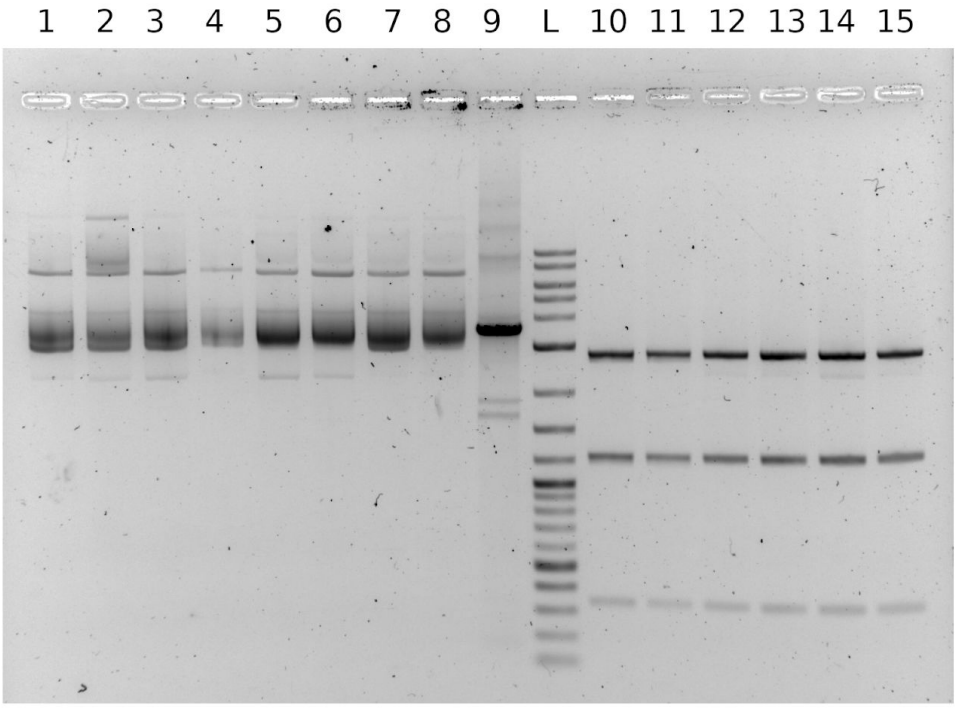
1) Prepare the 1% agarose gel
2) Prepare the samples : 2 µl of dye + 10 µl of digested plasmids

Results : the gel was not properly polymerized

Date 31st August

BbsI digestion
The digestion was realized following the same protocol.

Results :

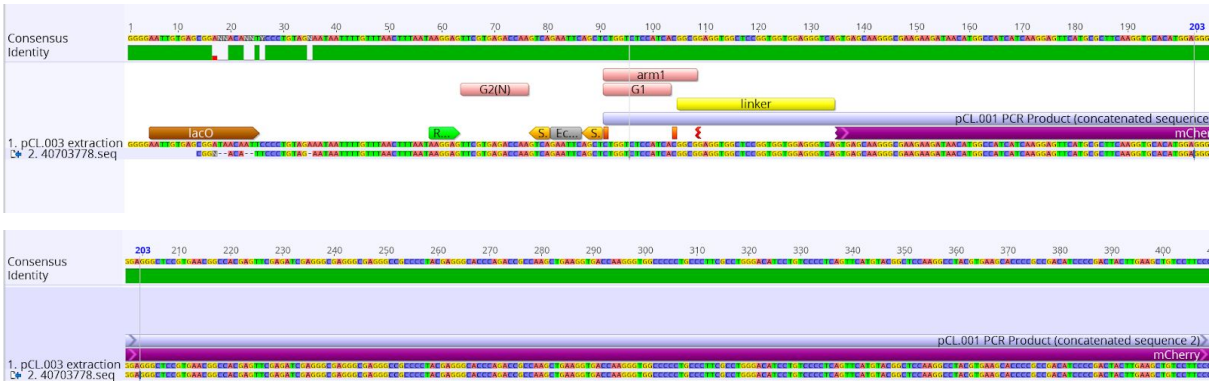


1-8 : Cold Fusion products non digested (colonies 1-8)
9 : pDuet plasmid non digested

L : ladder
10-15 : Cold Fusion products after digestion (colonies 1-6)

Date : 3rd September

Sequencing of the pDuet-mCherry plasmid (pCL003)



Clone 3 has been confirmed to contain the right sequence upstream mCherry and the correct sequence of mCherry. This plasmid will be used for further steps of the library assembling.

Date : 4th September

Twist Library Preparation (Provided quantity is 183ng)

1. Centrifuge the library (the tube containing lyophilized DNA).
2. Add 18 ul of dH2O to powdered DNA library.
3. Incubate it at room temperature for five minutes.

PCR of TWIST library, (from TWIST 7th August '18) **using ULTRA Q5 HF polymerase:**

ULTRA Q5 HF Polymerase was used in order to keep intact the library diversity and avoid DNA replication induced mutations. This is also why only 6 cycles of PCR were performed for the library amplification.

Note : Check the DNA concentration before PCR **183 ng.**, *Please read this Ultra Q5 PCR protocol, 8 reactions of 50ul*

Set up 8 reactions of PCR of 50 ul in following way :

Component	Volume (in ul)
NEBNext Ultra II Q5 Master Mix	25
10 uM forward primer	5
10 uM reverse primer	5
Template DNA	0.25
Water nuclease free	Upto 50

Note : Thaw the master mix gently and maybe quick spin of 5 seconds after thaw is recommended.

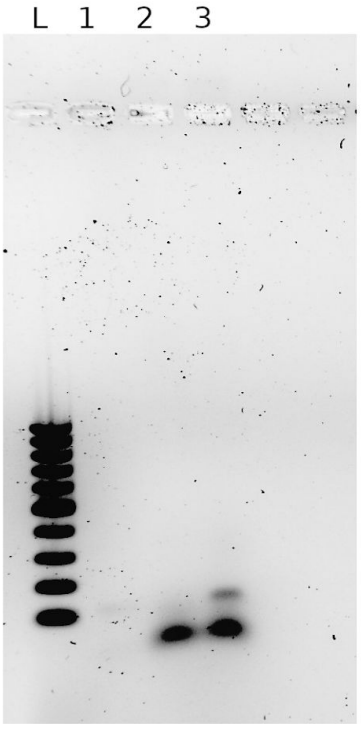
2) Thermocycling conditions for routine PCR

Step	Temperature	Time
Initial Denaturation	98℃	30 seconds
5 cycles	98℃	10 seconds
	61℃	30 seconds
	72℃	20 seconds
Final Extensions	72℃	5 minutes
Hold	4℃ (4-10℃)	∞

PCR cleanup was performed using a PCR purification kit(Promega).

Nanodrop quantification after purification : 70 ng/µl

1% Agarose Gel



L : ladder
1 : control PCR
2 : library PCR after PCR cleanup
3 : library PCR not purified

Results : The library amplification and the PCR clean up were successful.

Date : 7th September

Golden Gate Assembly of TWIST library with the sequenced pCL003.

Set up 8 reactions of 20 ul :

Component	Volume (in ul)
BsaI HF (20 000 U/ml)	1
T4 ligase (1U/µl)	1
ATP 10 mM (1mM final)	2
Cutsmart buffer 10X	2

Vector (75 ng final)	0.6
Insert (20 ng final)	0.3
Water nuclease free	Upto 20

Vector = pCL003, 120 ng/µl
TWIST Library = 70 ng/µl
Molar ratio insert : vector is 10:1

Step	Temperature	Time	Cycle
Digestion	37°C	5 min	60X
Ligation	16°C	5 min	
Inactivation	55°C	5 min	
Hold	12℃ (4-10℃)	∞	

The Golden Gate assembly of the library was done overnight, and the tubes have been put in the fridge during few hours. Before cleaning step, the GG mixture has been heated 5 min at 55°C to digest every remaining plasmid that did not have library insert by BsaI.

Date : 13th September

PCR cleanup of GG mixture (Promega)

Nanodrop quantification : 260/280 ratio looked to high (around 5), the absorbance curve was shifted but the shape looked normal.

Digestion of the GG (Golden Gate) and pDuet-mCherry plasmids with by BbsI
Master Mix 3*9 µl

Component	Volume (in ul)
BbsI HF (20 000 U/ml)	1
10X CutSmart buffer	3
H20	23

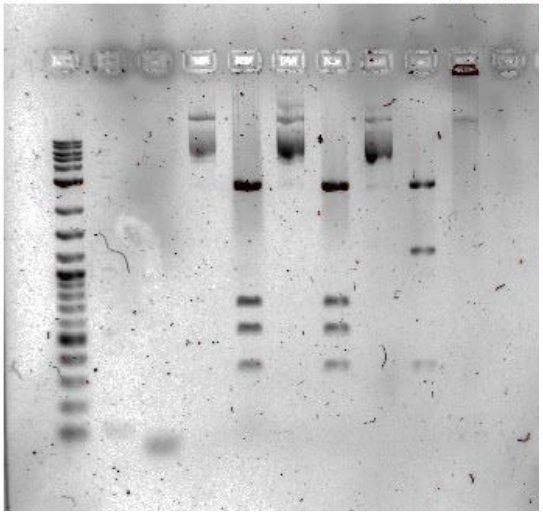
Final DNA concentration : 100 ng/µl

For pCL003 : 1.3 µl

For GG : 1µl

Incubation 40 min at 37°C without shaking

1% Agarose Gel



1 : Ladder

4, 6 : GG

5,7 : GG + Bbs I

8 : pCL003 + Bbs I

9 : pCL003

LB broth and LB agar preparation

Construction of an expression control for the pDUET plasmid containing mCherry with a short peptidic extension at N-term.

The peptidic extension (CLo10) is a neutral amino acid sequence to replace the library and quantify the expression of mCherry.

Gradient PCR of CLo10, 8*25µl

Component	Volume (in ul)
PCR buffer 10X	20
10 mM dNTP	4
10 uM forward primer	10
10 uM reverse primer	10
Template DNA	0.6
Taq DNA pol 5 U/µl	1.2
Water nuclease free	Upto 200 (154.2)

Step	Temperature	Time
Initial Denaturation	94℃	30 seconds
25 cycles	93℃	45 seconds
	47-57℃	60 seconds
	72°C	30 seconds
Final Extensions	72℃	10 minutes
Hold	4℃ (4-10℃)	∞

Date : 14th September

Gel verification of gradient PCR

Results : every temperature worked, probably wrong template concentration before

PCR cleanup of all tubes (Promega).
Nanodrop quantification : 20 ng/µl

GG plasmid send for sequencing

Golden Gate assembly of CLo10 with sequenced pCL003, 20µl*1 reaction

Component	Volume (in ul)
BsaI HF (20 000 U/ml)	1
T4 ligase (1U/µl)	1
ATP 10 mM (1mM final)	2
Cutsmart buffer 10X	2
Vector (75 ng final)	0.6
Insert (20 ng final)	1
Water nuclease free	Upto 20

Vector = pCL003, 120 ng/µl
CLo10 = 20 ng/µl
Molar ratio insert:vector : 20:1

Step	Temperature	Time	Cycle

Digestion	37°C	1 min	30X
Ligation	16°C	1 min	
Inactivation	55°C	5 min	
Hold	12℃ (4-10℃)	∞	

Transformation of NEB cells by CLo10 GG product

No purification has been done given that NEB cells are chemically competents.

1. Thaw NEB cells on ice, take 20µl of cells and add 2µl of CLo10 GG reaction
2. Incubate on ice 30 min
3. Heat the cells for 30 sec at 42°C
4. Put cells on ice for 5 min
5. Add 180 µl of cold SOC medium
6. Incubate 1h at 37°C with shaking
7. Plate 100 ul on appropriate LB-agar plates (50 µg/mL of Spectinomycin)
8. Incubate o/n at 37° C

Electroporation of NEB 10 beta HE cells by TWIST library GG reaction and pCL003 (positive control)

1. Sterilized 4*250ml erlenmeyers
2. Pre warm 2* 15 ml Falcon tubes, NEB 10-beta/Stable Outgrowth Medium and 10 Strep plates (50 µg/mL) at 37°C.
3. Place 2* electroporation cuvettes (2 mm) and 2 eppendorf tubes on ice.
4. DIlute pCL003 to 5 ng/µl
5. Add 5ng (in 1µl) of pCL003 and TWIST GG reaction in the 2 cold eppendorf tubes.
6. Thaw NEB 10-beta Electrocompetent cells on ice (about 10 min) and mix cells by flicking gently. Transfer 40 µl of the cells in the eppendorf tubes containing DNA.

7. Carefully transfer the cell/DNA mix into a chilled cuvette without introducing bubbles at the bottom of the cuvette.
8. Electroporate using Ec2 program.
9. Immediately transfer the cells in the Falcon tube containing 960 µl of 37°C NEB 10-beta/Stable Outgrowth Medium.
10. Shake vigorously (250 rpm) or rotate at 37°C for 1 hour.

Plating of NEB cells containing CLo10 GG product

The entire volume (200µl) has been plated on a Spec plate and incubate at 37°C overnight

Plating of NEB 10 beta HE cells containing first TWIST library GG product and pCL003 (positive control)

1. Take 20µl of the electroporation mixture for serial dilution
2. Split the 980µl left by transfering 245µl of the electroporation mixture in the 4 erlenmeyers containing 50 ml LB broth + 50µg/ml Strep and put them at 37°C with shaking overnight
3. Dilute the remaining 20µl with 180µl of LB broth
4. Plate 100µl on a Spec plate (Dilution 1)
5. Make serial dilutions (Dilutions 2-5) with 900µl of LB broth + 100µl of culture
6. Plate 100µl of each dilution on Spec plates
7. Incubate plates at 37°C overnight

Date : 15th September

Glycerol Stock of each overnight liquid culture of electroporated cells (with TWIST GG reaction : 4*50ml) and freezing (at -20°C during 10 hours and -80°C)

Centrifugation of the 4*50 ml overnight culture, freezing of cell pellets (-20°C)

Cell counting :

- CLo010 : 592 colonies
- Positive control (pCL003)

    - Dilution 5 : 69
    - Dilution 4 : 235
    - Dilution 3 : 1200
    - Dilution 2 and 1 : too much
- Twist library :
    - Dilution 5 : 4
    - Dilution 4 : 42
    - Dilution 3 : 346
    - Dilution 2 and 1 : too much

Inoculations of 5 clones from CLo10 and twist library plates in 5* 50ml Falcon tubes containing 10 ml of LB Strep (10µg/ml). Incubation overnight at 37°C without shaking.

Date : 16th September

Glycerol stock preparation of colonies from CLo10 and twist library inoculations.

Miniprep of CLo10 cultures (2 samples), twist cultures (2 samples) and frozen library pellet coming from the 200 ml liquid culture.

Nanodrop quantification after miniprep

CLo10 1 : 137 ng/µl
CLo10 2 : 107 ng/µl
Twist 1 : 37 ng/µl
Twist 2 : 18 ng/µl
Library 1 : 15 ng/µl
Library 2 : 139 ng/µl

The sample CLo10 2 send for sequencing

Glycerol Stock from the library overnight culture and storage at -80°C

Miniprep extraction from part of library overnight culture (A&A Biotech, plasmid mini kit)

1. Transfer 100 ml of the overnight culture in 2*50 ml falcon tubes
2. Centrifuge 10 min at 4500 rpm
3. Remove the supernatant and resuspend the pellet in 1 ml of L1 buffer
4. Transfer the lysate in 4 eppendorf tubes
5. Add 500 µl of L2 buffer per tube, mix gently and incubate 3 min at RT
6. Add 1 ml of GL3 and mix until disappearance of raspberry colour
7. Centrifuge 2*10 min at 14 000 rpm, discard the filtrates
8. Add 500 µl of W buffer and centrifuge 1 min at 14 000 rpm, discard the filtrates
9. Add 600 µl of A1 buffer and centrifuge 2 min at 14 000 rpm, discard the filtrates
10. Wait until ethanol evaporation
11. Transfer the columns into dry eppendorf tubes
12. Elute with 50µl of sterile water

Nanodrop quantification : 240 ng/µl (final volume =200µl)

BsaI digestion of purified library plasmid

Digestion of 3µg of the miniprep product

1. Master mix preparation for 3*50µl

DNA (240ng/µl) : 12.5 µl
Cut-smart Buffer (10X) : 15 µl
BsaI HF (20 000U/ml) : 1.5 µl
H20 : 121 µl

2) Transfer 50 µl in 3 tubes
3) Incubate 1h at 37°C and 300 rpm

Purification of the digested library samples (Wizard® SV Gel and PCR Clean-Up System, Promega)

Elution in 20 µl

Preparation of 1 sample to run on a 1% agarose gel (0.25µl DNA, 1µl dye, H20 qsp 6µl)

Storage at -20°C

Date : 23th September

Transformation of mCherry control, GG1 in normal BL21 cells

-2*50µl of XJ autolysis cells
-40 ng of control
-40 ng of GG1
-10 Spec plates
-LB = 2ml + 5ml (for serial dilution)

1. Pre-warm plates at 37°C one for each transformation.

2. Thaw BL21 Competent cells on ice. (from Haotian, aliquot of ~500µl, 20 min)

3. Add 40 ng to 50ul of cells and gently mix
4. Incubate on ice for 10 minutes.
5. Heat-shock the transformations by placing the tubes in a 42°C for 30 seconds.
6. Place on ice 2 minutes.
7. Add 1ml of LB to the transformation and incubate for 1 hour at 37°C with shaking at 200-300RPM
8. Do a serial dilution:
9. Spread 100 µl of the different dilutions on Spec plates (5 plates for GG1 and 4 plates for mCherry, not the 10^-1)
10. Strike on a plate without antibiotic trawed BL21 cells
11. Incubate the plates at 37°C overnight
12. From the 1ml transformed culture of GG1 and control, take 50µl and add it to 500µl of LB Spec in 2ml eppendorfs and incubate tubes overnight at 37°C with shaking 250 rpm.
13. Inoculate BL21 cells in 500 µl of LB in 2ml eppendorf and incubate it overnight at 37°C with shaking 250 rpm.

Auto-induction media preparation

Autoinduction media:

	/1L
- Phosphate Buffer (pH 7.2)	6g Na_2HPO_4/3g KH_2PO_4
- Tryptone	20 g
- Yeast Extract	5 g

- NaCl 5 g

Preparation of 1L twice, pH adjustment
Split the 1L into 6 bottles of 250ml (200ml/bottle)
Autoclave the bottles

Warning : before using the media, add :

- 60 % v/v Glycerol 10 ml
- 20 % w/v Glucose 2.5 ml
- 20% w/v Arabinose 2.5 ml
- 100 mM IPTG 1 ml
- Spectinomycin (50 mg/ml) 50 µg/ml

Date : 24th September

Colony counting

GG1
-10^-1 : 130
-10^-2 : 66
-10^-3 : 8
-10^-4 : 2
-10^-5 : 0

mCherry control
-10^-2 : 8
-10^-3 : 13
-10^-4 : 0
-10^-5 : 0

Plating of 40 Spec plates

Date 28- 2/10

Expression test

1. Pick 2 colonies of the mCherry control, 2 colonies of one variant (called GG1) and 2 colonies of WT XJb cells.
Do an overnight culture in 10 ml of LB-Spectinomycin, except for the WT cells (10 ml LB)
2. Check the OD600 in the next morning,
3. Start new cultures in 5 ml of LB-Spectinomycin, by adding the equivalent of OD=0.05.

Incubate the cultures at 37°C with shaking.
4. When the OD reached 0.4 (around 1h30 of incubation), induce them by adding 0.1mM or 0.5 mM of IPTG and incubated them at 28°C or 37°C overnight
The induction was done as well when OD=0.8 (around 3h of incubation) with both IPTG concentrations and expression temperatures.
None of them worked.

Date 3/10

12h culture of each strain

Induction test in 96 well plates

Two plates was done by following this plan

1. Add 150 µl of LB in line C1-C6, F1-F6 and G1-G12
2. Add 150 µl LB Spec in line A1-A6, B1-B6, D1-D6, E1-E6
3. Add AI media IPTG 0.1 mM in C7-C9, F7-F9
4. Add AI media IPTG 0.5 mM in C10-C12, F10-F12
5. Add AI media IPTG 0.1 mM Spec in B7-B9, C7-C9, D7-D9, E7-E9
6. Add AI media IPTG 0.5 mM Spec in B10-B12, C10-C12, D10-D12, E10-E12
7. Add AI media in line H
8. Add 2.75 µl of each strain
9. Incubate 1:30 at 37°C
10 : Add 1.5 µl IPTG 100mM in A1-F3
11. Add 7.5 µl IPTG 100mM in A4-F6
12. Incubate ON (Induction start at midnight

	1	2	3	4	5	6	7	8	9	10	11	12		
A	XJ GG1 (1)	XJ GG1 (2)	XJ GG1 (3)	XJ GG1 (1)	XJ GG1 (2)	XJ GG1 (3)	XJ GG1 (1)	XJ GG1 (2)	XJ GG1 (3)	XJ GG1 (1)	XJ GG1 (2)	XJ GG1 (3)		
B	XJ CTR L (1)	XJ CTR L (2)	XJ CTR L(3)	XJ CTR L (1)	XJ CTR L (2)	XJ CTR L(3)	XJ CTR L (1)	XJ CTR L (2)	XJ CTR L(3)	XJ CTR L (1)	XJ CTR L (2)	XJ CTR L(3)		LB IPTG 0.1 mM
C	XJ WT (1)	XJ WT(2)	XJ WT (3)	XJ WT (1)	XJ WT(2)	XJ WT (3)	XJ WT (1)	XJ WT(2)	XJ WT (3)	XJ WT (1)	XJ WT(2)	XJ WT (3)		LB IPTG 0.5 mM
D	BL21 GG1(1)	BL21 GG1(2	BL21 GG1 (3)	BL21 GG1(1)	BL21 GG1(2	BL21 GG1 (3)	BL21 GG1(1)	BL21 GG1(2	BL21 GG1 (3)	BL21 GG1(1)	BL21 GG1(2	BL21 GG1 (3)		AI IPTG 0.1 mM
E	BL21 CTR L (1)	BL21 CTR L (2	BL21 CTR L(3)	BL21 CTR L (1)	BL21 CTR L (2	BL21 CTR L(3)	BL21 CTR L (1)	BL21 CTR L (2	BL21 CTR L(3)	BL21 CTR L (1)	BL21 CTR L (2	BL21 CTR L(3)		AI IPTG 0.5 mM

F	BL21 WT(1)	BL21 WT(2)	BL21 WT(3)	BL21 WT(1)	BL21 WT(2)	BL21 WT(3)	BL21 WT(1)	BL21 WT(2)	BL21 WT(3)	BL21 WT(1)	BL21 WT(2)	BL21 WT(3)		
G	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB		
H	LB	LB	LB	LB	LB	LB	AI	AI	AI	AI	AI	AI		

AI media preparation

For 15ml :
-Glycerol 60% = 150 µl
-Glucose 20% = 37.5 µl
-Arabinose 20% = 37.5 µl
-IPTG 100 mM = 15 µl (0.1mM) OR 45µl (0.5mM)

Date 4/10

OD/fluo measurement at 12:00, 18:00 and 20:00

Results : The best induction time is 12h

Freeze the two plates at -80°C

Incubate overnight and measure the growth curve of E. coli

E coli overnight culture
Pick one colony from the plate and inoculate 5 ml of LB

Date 5/10

E coli culture
Add 125 µl in 5 ml of LB and incubate 2h
Take 1 ml, centrifuge 5 min at 10 000 rpm
Wash the pellet with 1 ml of PBS
Resuspend the pellet with 1 ml of LB
Dilute with LB to have OD ~ 0.01

Thawing of cell = lysis step

In parallel of plate preparation, thaw the plates (stored at -80°C) at RT during 10-15 min.

Keep it at 4°C after thawing.
Centrifuge the plates (upstairs) 10 min at 6 000 rpm (4°C)

Preparation of killing assay plates

-6* 96 well plates was made following these steps:

Add 150 µl of LB in each well
Add 2µl of diluted E coli culture in each well except last line
Incubate at 37°C for 4h (one plate on the reader to check the growth curve)

In parallel, thaw the two plates (of 3/10) at RT (15:30) during 45 min and keep them at 4°C

-1 plate (plate 7) was made following these steps:
Add 150 µl of LB Spec in line A, B, D, E
Add 150 µl of LB in line C, F, G

Killing assay

After centrifugation, measure the OD and fluo of the 6 plates containing the E. Coli culture. They should be in the log phase (around 4 hours of growth, OD~0.2-0.3 in the Tecan).

Only the Plate 2 (of induction) was used. The plate 4 was stored at -20°C)
Plate 1 = add 2 µl of lysate from induction plate
Plate 2 = add 5µl of lysate from induction plate
Plate 3 = add 10µl of lysate from induction plate

Resuspend the cell pellet by pipetting up and down
Plate 4 = add 10µl of lysate from induction plate
Plate 5 = add 5µl of lysate from induction plate
Plate 6 = add 2 µl of lysate from induction plate

Plate 7(the plate with only LB and LB spec) = add 15µl of cells
→ to see if cells can grow in LB after lysis
In well G1-3 : add again 15µl of XJ WT (AI 0.1)
In wells G4-6 : add 15µl of BL21 WT (AI 0.1)
Add 20 µg/µl of Ampicillin in these 6 wells : positive control of death

Induction plate (plate 2)
→ To see if cells can grow with addition of glucose after lysis

Add 50 µl of glucose 0.015% in each wells

Incubate every plates at 37°C with shaking for at least 4h
Measure both OD and fluo

Date 09/10

Colony picking (around 16:30-19h)
Preparation of 50 deep 96 well plates with 500 µl of LB + spectinomycin
Picking of 5000 colonies with toothpicks
Sealing with plastic films
Overnight culture without shaking, 37°C

Date 10/10

Induction of protein production (around 13h)
Prepare 50*96 well plates with 150µl of autoinduction media
Add 500 µl of glycerol 30% in each wells of the overnight plates
Replicate each overnight plates in the induction plates by using a pin tool
Seal the induction and overnight plates with aluminium films
Store the overnight plates at -20°C
Incubate the induction plates 12h at 37°C
Freeze the induction plates at -80°C

Colony picking of the control plate

Date 11/10

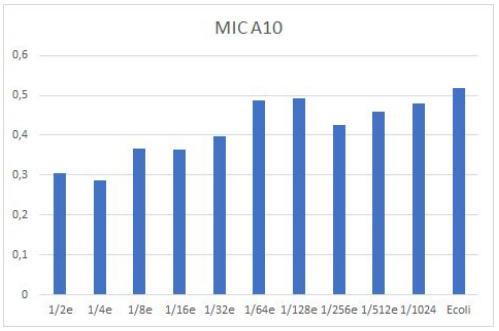
Killing assay and recovery (line 7 of plate 21B)
E coli culture (1:30)
PBS wash
Pellet resuspension in 1ml of LB
Dilution until 0.01

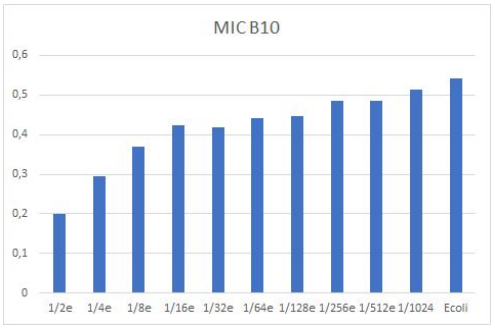
In a 96 well plate, add 50µL of LB + 50 µl of lysate (from 21B)
Do a serial dilution (50ul) for column 1 to 10
Add 1µl of E coli by pin tool, except in column 12
Incubate 12h in the Tecan

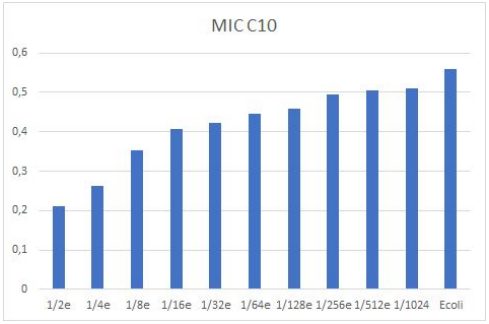
For the recovery plate, add 10 µl of lysate into 90 µl of LB + 1% glucose
Incubate 12h in the Tecan

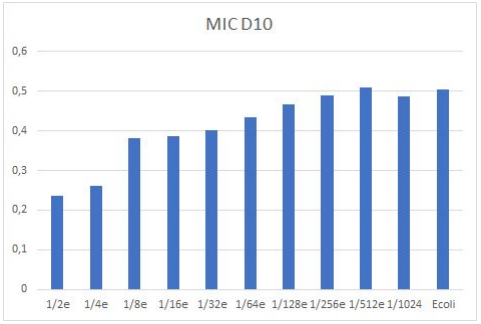
Results:

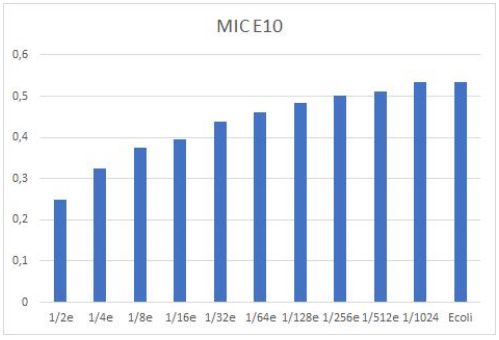
-MIC determination of the 8 variants at 12h

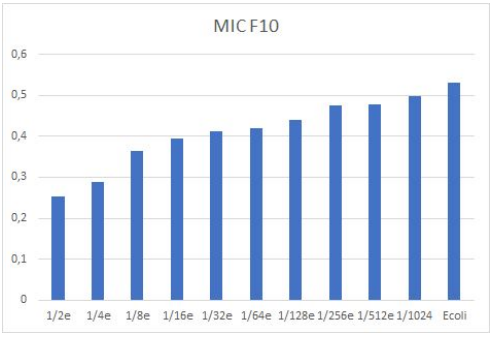


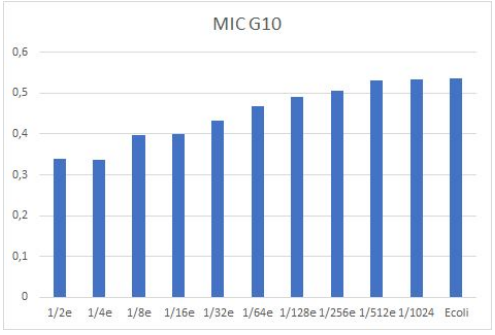


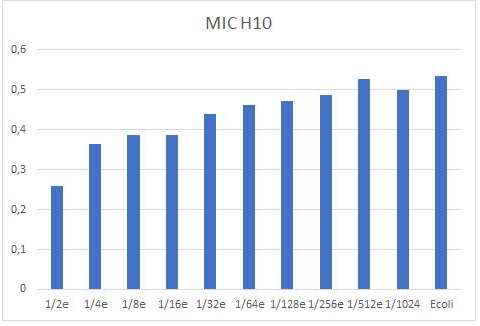




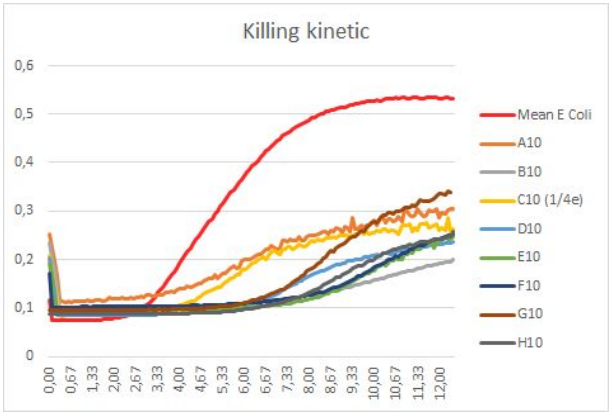








- Killing kinetic graph :



Results : In order to discriminate de killing properties of our variants, 8h of incubation seems a good choice.

Induction of the control plate

Prepare a 96 well plates with 150µl of autoinduction media in the lines A, B, C
Add 500 µl of glycerol 30% in each wells of the overnight plates
Replicate each overnight plates in the induction plates by using a pin tool
Seal the induction and overnight plates with aluminium films
Store the overnight plates at -20°C
Incubate the induction plates 12h at 37°C
Freeze the induction plates at -80°C

Date 13/10 and 14/10

Killing assay of the 50 plates

E coli culture (1:30)
PBS wash
Pellet resuspension in 1ml of LB
Dilution until 0.01 in 40 ml

In 96 well plates, add 75µL of LB + 25 µl of lysate (1/4e dilution)
Add 1µl of E coli by pin tool
Incubate 8h at 37°C
During incubation, measure OD and fluorescence of the lysate plate that contain the 125ul remaining lysates
After incubation, measure OD600.

The killing assay was done in 3 rounds : 10 plates, 15 plates and 25 plates.
One control plate was realised each time to normalized the results.

Date 14/10

Data analysis

Date : 16/10

PCR of the 182 best samples

Master mix for 100 reactions of 20ul :

Component	Volume (in ul)
Dream Taq polymerase	1ml
10 uM forward primer	100
10 uM reverse primer	100
Water nuclease free	Upto 2ml

Step	Temperature	Time
Lysis step	95°C	5 min
Initial Denaturation	95℃	30 seconds
25 cycles	95℃	30 seconds
	50℃	30 seconds
	72°C	30 seconds
Final Extensions	72℃	5 minutes
Hold	4℃ (4-10℃)	∞

-add 20 µl of the mastermix in each well of a 96 well plates
-pick the correct samples from glycerol stock and inoculate the wells
-seal the plates with a plastic cover