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Avani Koparkar

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100 mL
2.5 g

150 mL
3.75 g

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- Preparation of LB medium

Materials: LB broth, Miller Ref. M1245-500G.
- Himedia. 500g.

In 1L autoclave bottle :-

Taken ↓

25g LB broth powder → 6.25g
- 2.5g taken

1000mL ultrapure water → 250mL
- 100mL taken

↓

Swirl to mix → Dissolving not necessary

↓

Replace the cap to the bottle but leave it slightly loose for pressure equilization. Place a Fresh piece of autoclave tape

↓

Autoclave (121C. 20 min)

↓

Cool to RT before use. Do not tighten the cap until cool.

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15g - 1L 150 100mL
? 250mL 2.25g 1.5g

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Preparation of LB-Agar plates ~30/35 plates
Want :- 10 plates

Materials: LB medium, ~~agar~~ plates (~30-35 plates) Erlenmeyer Flask, 1.5% agarose
Bacteriological agar: RM026-500G, Himedia (500g)

250mL
1L of LB liquid (25g broth powder per 1L) in a 2000mL E.m Flask

250mL

↓

Add ~~1.5%~~ 15g (1.5%) ~ 4g taken 3.75g
bacteriological agar

↓

Swirl to mix. Powder will not dissolve completely

↓

Add a Fresh piece of aluminium Foil + Fresh piece of autoclave tape

↓

Autoclave (121C 15 min.)

↓

Remove to benchtop to cool to ~50C

↓

Add antibiotics (if any)

↓

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35 plates

250mL

Set your plates & label appropriately

↓

Pour out the media into petri plates :-
- Remove lid → Remove Foil → Pour enough LB agar to cover the bottom

↓

All within 5 mins

↓

Pour plates in small stacks of 4-5 plates & stack up to 10 plates together

↓

Store plates back in plastic ~~sle~~ sleeve
Store upside-down.

For 250ml
3.75 g agar
6.25 g LB powder

For 200ml
5g LB powder
3g agar

1000mL
25g LB powder
15g agar

For 100mL
2.5g LB powder
1.5g agar.

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SOB & COMBRO.

250 ml SOB

1. 1.25g yeast extract
2. 5g tryptone
3. 0.146g NaCl
4. 0.0465g KCl
5. 1.2325g $MgSO_4 \cdot 7H_2O$

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Competent cells: iGEM

~~35ml~~ 50ml SOB

Innoculate 5ml of SOB medium with 200µl seed stock & grow at 20°C. OD600nm of 0.3

- Takes 16 hrs
- Works at room temp
- Aim for lower OD, not higher

Incubated for +2 hrs 20°C

Pre-chill as many Flat-bottom centrifuges as you can in an ice buckets

Transfer the culture to Flat-bottom centrifuge. Weigh & balance the tubes on a scale (As close as possible, $\Rightarrow \Delta w = 1g$)

Centrifuge at 3000g at 4°C for 10 min in a Flat centrifuge bottle

Decant supernatant into waste receptacle

Gently resuspend in ~~80~~ 1.6ml of (320µl per eppy) ice cold CCMB80 buffer (Add ½ & ½)

Swirl gently

Incubate on ice ~~at~~ for 20 minutes

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centrifuge again at 3000g at 4°C.
Decant & dispose supernatant
↓
Resuspend cell pellet in ~~2ml per copy~~ 40µl of ice cold CCMB80 buffer
2ml
↓
If using Flat bottom centrifuges, combine cells post-resuspension
↓
Use Nanodrop to measure OD of a mixture of ~~4~~ 160 µl LB & 40µl of resuspended cells. Use a mixture of ~~4~~ 160 µl LB & 40µl CCMB80 buffer as the blank
↓
Add chilled CCMB80 to yield a Final OD 1.0 - 1.5 in this test
↓
Incubate on ice For 20 min. Prepare For aliquoting
↓
Aliquot into chilled 2ml microcentrifuge tubes ~~of 50µl~~
↓
Store at -80°C indefinitely
↓
Test competence.

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Measurement of Competence

Transform 50 µl of cells with 1 µl of standard pUC19 plasmid

↓

This can be made by diluting 1 µl of NEB pUC19 plasmid into 100 ml of TE (can use water)

↓

Incubate on ice 0.5 hrs. Pre-heat water bath now

↓

Heat shock 60 sec at 42°C

↓

Add 250 µl LB

↓

Incubate at 37°C for 1 hr in 2 ml centrifuge tubes, using a mini-rotator

↓

C & T resistant : 2 hrs
Amp & Kan : 1 hr

↓

Add 4-5 sterile glass beads to each agar plate, then add 20 µl of transformation

↓

After adding, gently move plates from side to side to redistribute beads. When most of it is

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Use 3 plates per vial tested

↓

Incubate plates agar-side up at 37°C for 12-16 hrs

↓

Count colonies on light field the next day

↓

Good cells should yield 100-400 colonies.

Transformation efficiency is :-

Dilution Factor = 15 X colony count X 10^5 / µgDNA

↓

Expected transformation efficiency should be between 1.5×10^8 & 6×10^8 cFu / µgDNA.

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Transformation.

Materials:

1. Resuspend DNA: with 10µl dH_2O. Pipet up & down several times & let sit for a few minutes; turns RED

2. 10pg/µl control DNA: 1µl for control transformation pSB1C3 on a high-copy Chloramphenicol plasmid

✱ Use lab timer & thermometer ✱

Thaw comp cells on ice.

↓

Pipette 50µl of competent cells into 1.5ml 2ml tube which are labeled. prechilled. Make 1.5ml 2ml tube for control also

↓

Pipette 1µl of resuspended DNA into 1.5ml tube. Mix gently

↓

Pipette 1µl of control DNA into 1.5ml tube. (10pg/µl (10ng/µl))

↓

Close ~~2ml~~ 1.5ml tubes, incubate on ice for 30 min

↓

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Heat shock tubes at 42°C for 1 min

↓

Incubate on ice for 5 min

↓

Pipette 200 µl SOC media to each transformation (SOC replaced by LB)

↓

Incubate at 37°C for 2 hrs (shaker)

↓

Pipette each transformation on two petri plates for a 20 µl & 200 µl plating

↓

Incubate transformations overnight (14-18 hr) at 37°C

↓

Pick single colonies

↓

Count colonies for ~~te~~ control transformation.

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Seed stock prep

15% glycerol

Total vol = 1ml

∴ 150 µl Glycerol } 1 eppy
850 µl cells }

150 ml SOB	150ml SOB.
1. 0.5% w/v yeast extract.	0.075g
2. 2% w/v tryptone	3g
3. 10mM NaCl	0.0876g
4. 2.5mM KCl	0.0279g
5 20mM $MgSO_4 \cdot 7H_2O$	0.7395g

246.5 $MgSO_4 \cdot 7H_2O$

$246.5 \times 20 \times 10^{-3}$ 1000mL
? 150

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Inoculation time : 12:52 am

A 600nm

0.515 A MG1655 0.509, 0.500 10.53 am

0.055 A DH5α 10.54 AM

16/5/17

Inoculating the main culture (again)

3 - DH5α

2 - MG1655

MG1655 : 2 X 5mL + 8mL + 7mL = 25mL SOB (extra, blank)
Inoculate with 100µL seed stock

DH5α : 3 X 5mL + 8mL + 7mL = 30mL SOB
Inoculate 120µL DH5α seedstock

(20µl in 5 ml)

Measure the above volumes of SOB into 250ml Scott bottles

↓

Inoculate with above mentioned volumes of seed stock

↓

Grow @ 20°C @ 280rpm For 18 hrs

↓

Measure OD periodically.

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Readings

Inoculation time: 12:52 am

A	600 nm	
0.515	MG1655	10.53 am
0.055	DH5α	10.54 am.

↓ Want: 0.3 OD

Dilution of MG1655 1:1
(Add 23.5 ml more; grow for 1 hr)

OD readings of MG1655

A		
	0.259	12.07
0.357	We diluted it 2:1	At inoculation
0.313	After 20 mins, 2:1 dilution.	~~½ hr~~ 15 min
		1 hr

DH5α

0.055	10.54 am
0.102	12 noon
~~0.132~~	1.05 pm.
0.148	2.10 pm
0.205	3.10 pm

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OD @ 1655

Avani : 1.111 ✓ 1 to 1.5 range

Jyothish : 1.355 1 to 1.5 range

This is after taking 650 µL LB & 150 µL culture.

Dilution Factor = $\frac{650}{150} = \underline{4.33}$

OD_A = 1.111 × 4.33 = 4.810

OD_J = 1.355 × 4.33 = 5.867

300µl remaining. (per person)

⇒ Use 1300 µl ~~LB to #~~ CCMB80 to dilute.

Avani : 0.270

Jyothish : 0.418

Dil. f.

OD_A = 0.270 × 5 = 1.350 ✓

OD_J = 0.418 × 5 = 2.090

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SOB 25ml

1. 0.125g yeast extract
2. 0.5g tryptone
3. 0.0146g NaCl
4. 0.00465g KCl
5. 0.12325 $MgSO_4 \cdot 7H_2O$

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Competent Cell Test Kit.

Clean your work area by wiping with 70% ethanol

↓

Thaw comp cells on ice. Label one 1.5mL microcentrifuge tubes per transformation & then prechill on ice.

(Do triplicates (3 each) of each conc. if possible)

↓

Spin down the DNA tubes from the Comp cell test kit to collect all of the DNA into the bottom of each tubes (20-30s at 8,000 - 10,000 rpm)

↓

Pipet 1 µL of DNA into each microcentrifuge tube

↓

Pipet 50 µL of comp cells into each tube. Flick the tube gently with your finger

↓

Incubate on ice For 30 min.

Pre-heat waterbath now to 42°C.

Otherwise, hot water & thermometer works!

↓

Heat-shock the cells by placing the waterbath for 45s (<1 min) Be careful to keep the lids of tubes above water level, & keep ice close by

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↓

Immediately transfer the tubes back to ice, & incubate on ice for 5 min

↓

Add 950 µl of LB media per tube & incubate at 37°C for 2hr shaking at 200-300 rpm

↓

Pipet 100 µL from each tube into the appropriate plate & spread the mixture evenly.
Incubate at 37°C overnight (~16 hrs)

↓

Count the no of colonies
(Use avg colony count)
Efficiency (in cfu/µg) = [colonies on plate (cfu) / Amount of DNA plated (ng)] x 1000 (ng/µg)

Amnt of DNA plated = How much DNA plated on the agar plate.

Amount of DNA plated (ng)
= Vol. of DNA added (1 µL) x
conc. of DNA (& convert to ng/µL) x
[Volume plated (100 µL) / total reaction volume]

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MG1655 growth curve

Absorbance readings: Check diameter

Inoculation : 5 am $\lambda = 615$ nm

	Time	Abs	0.43 1.30
~~2~~			0.543 2.0
2.	9.30 am	CF x 0.04	
3.	11.15 am	CF x 0.08	
4.	12 noon	CF x 0.10	
5.	1 pm	CF x 0.18	
6.	1.30 pm.	CF x 0.21	
7.	2.00 pm	CF x 0.26	
8.	2.38 pm	CF x 0.33	
9.	3.05 pm	CF x 0.37	
10.	4.05 pm	CF x 0.37	

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Calculations for $CaCl_2$ method :-

① $MgCl_2$ - $CaCl_2$ soln

Wanted :- 100ml

• Weight of $CaCl_2 \cdot 2H_2O$ (20mM)

$$146.98 \times 20 \times 10^{-3} \times \frac{100\ ml}{1000\ ml}$$

$$= 0.29396\ g$$

• Weight of $MgCl_2 \cdot 6H_2O$ (80mM)

$$203.3027 \times 80 \times 10^{-3} \times \frac{100\ ml}{1000\ ml}$$

$$= 1.626\ g$$

② $CaCl_2$ (1M)

Weight of $CaCl_2 \cdot 6H_2O$ taken (1M)

$$= 14.698\ g$$

($CaCl_2$ taken : 11.1g)

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$MgCl_2 \cdot 6H_2O$ MW = 203.3027

80mM in 100ml : 1.626

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25/5/17

Competent cell preparation: $CaCl_2$

Materials: 1) $MgCl_2 \cdot CaCl_2$ sol^n
80mM $MgCl_2$, 20mM $CaCl_2$
2) $CaCl_2$ sol^n : 0.1mM
3) LB : 150ml

$CaCl_2 \cdot 2H_2O$
20mM
0.29396
For 100ml

Calculation:

① $MgCl_2$-$CaCl_2$ sol^n 500ml

$MgCl_2$: $80 \times 95.211 \times \frac{500ml}{1000ml} \times 10^{-3}$

= 3.80844g ≈ 3.808g

For 100ml take 0.7616888g

② $CaCl_2$: 500ml

$20 \times 110.98 \times \frac{500}{1000} \times 10^{-3}$

= 1.1098g

For 100ml take = 0.22196g

③ $CaCl_2$ 0.1M in 50ml of sol^n

$= 0.1 \times 110.98 \times \frac{50}{1000} = 0.5549g$

Take 1% of secondary culture in LB of 50mL

(Have :- 10mL ∴ We take 100µl)

↓

Put the secondary culture at 37°C

↓

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Measure OD after 2 hrs

↓

(Take the culture & put it in 2 Falcon tubes equally (25ml & 25ml)) Not necessary for 10ml

↓

Put these in tubes at 0°C on ice for 10 mins

↓

Then centrifuge for 10 mins at 4°C & 4100 rpm

↓

Decant the tubes. Add 3ml 3ml ~~5ml + 5ml~~ $MgCl_2$- $CaCl_2$ sol^n. Resuspend gently ON ICE

↓

Put the tubes in 0°C (ice) for 45 mins

↓

Centrifuge the tubes for 10 min at 4°C & 4100 rpm

↓

Decant the tubes

200µl ↓

Put ~~100µl~~ (0.1M) $CaCl_2$ in both tubes

↓

Add 80% glycerol (40µl) in each tube

↓

Fill eppys with this sol^n (50µl)

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C V
8 2 50
? 100

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26/5/17

OD Track ~~DH5~~ MG1655 in LB at 37°C.

Inoculated: 120 µl in 12 ml LB

Time : 10.10

OD	Time
0.07	11.20
0.2	12.00
0.938	1.00

Result

46 transformed colonies (Pink)

$$TE = \frac{\text{No of colonies} \times \text{Total culture Vol}}{\text{Vol plated}} \quad \frac{\text{cfu}}{\mu\text{g DNA}}$$

$$\text{Conc. of DNA} \times \text{Vol. DNA} \quad (\text{pg of DNA})$$

$$= \frac{25 \times 1000 \times 10^6}{50}$$

$$\frac{100\ \text{pg}}{\mu\text{L}} \times 1\ \mu\text{L}$$

5

$$= \frac{25 \times 1000 \times 10^6}{5000} \quad \frac{\text{cfu}}{\mu\text{g DNA}}$$

2

$$= 5 \times 10^6\ \text{cFu}/\mu\text{g. DNA}.$$

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9/6/17

Transformation

① DH5α (I) ② DH5α (Ag) ③ MG1655 (I)

DNA :- 1µl 100 pg/µl

→ Heat shock:

ice incubation 35 min

Waterbath: 44°C 45 sec

Got colonies.

EFFICIENCY

DH5α (I)

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29/5/17

Bacterial growth curve.

Autoclaved 2 conical Flasks

↓

Took 50 mL SOB in both

↓

Inoculated one with 200 µL DH5α
& other with 200 µL MG1655

↓

- OD measurements.

Incubation: 20°C . 250 rpm.

Time	DH5α OD	MG1655 OD
8.15 pm	0.031 X Bad cuvette	0.008
8.35 pm	0.019	
9.10 pm	0.048	0.045
10.10 pm	0.053	0.048
11.10 pm	0.050	0.075
30/5 12.10 am	0.050	0.098
1.10 am	0.055	0.145
2.10 am	0.060	0.205
3.10 am	0.066	0.311
4.10 am	0.073	0.523
5.10 am	0.077	0.726 / 0.726
6.10 am	0.090	1.068
7.10 am	0.101	1.536
8.10 am	0.122	2.148
9.10 am	0.144	2.796
10.10 am	0.159	3.134

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11.10 am	0.176	3.920
12.10 pm	0.211	5.36
1.10 pm	0.257	5.120
2.10 pm	0.258	5.080
3.10 pm	0.319	6.280
4.10pm	0.417	10.50
5.10pm	0.457	8.00
6.10 pm	0.539	6.26
7.10 pm	0.613	6.52
8.10 pm	0.772	7.52

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Measurement Track

* Transformations done on 5/6/17
1. Positive control (BBa_I20270)
2. Negative control (BBa_R0040)
3. Test Device 1 (BBa_J364000)
4. Test Device 2 (BBa_J364001)
5. Test Device 3 (BBa_J364002)
6. Test Device 4 (BBa_J364003)
7. Test Device 5 (BBa_J364004)
8. Test Device 6 (BBa_J364005)

↓

PBS buffer made

↓

Fluorescein stock made from iGEM
(A Lab:- MW = 376.275 (Sodium Fluorescein)
100μM stock weight = 0.037627 5
~0.037 6 3
CA lab has Fluorescein.
M.W of ~~sodium~~ Fluorescein: 332.31
100μM stock in 1L = 0.033231
100mM stock in 1mL = 0.033231

↓

Dilute it 1000 times to get 100μM in 1ml

↓

We made 1M NaOH & did 11 dilutions of 1X Fluorescein (50μM)
0.033 g in 10 ml

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CA = Lab Fluorecein
(1), (2) = iGEM

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	2	3	4	5	6	7	8	9	10	11
A	50mM (1)	25mM (2)	12.5mM (3)	6.25mM (4)	3.125mM (5)	1.5625mM (6)	(7)	(8)	(9)	(10)
B	(11)	(12)								

	1	2	3	4	5	6	7	8	9	10	11	12
A		CA (1)	CA (2)	CA (3)	CA (4)	CA (5)	CA (6)	CA (7)	CA (8)	CA (9)	CA (10)	
B	CA (11)	CA (12)	CA (1)	CA (2)	CA (3)	CA (4)	CA (5)	CA (6)	CA (7)	CA (8)		
C	CA (9)	CA (10)	CA (11)	CA (12)	CA (13)	CA (2)	CA (3)	CA (4)	CA (5)	CA (6)		
D	CA (7)	CA (8)	CA (9)	CA (10)	CA (11)	CA (12)	CA					
E	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
F	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
G	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
H	CA 1	CA 2	CA 3	CA 4	CA 5	CA 6	CA 7	CA 8	CA 9	CA 10	CA 11	CA 12

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	"	"	"	"	"							
C	"	"	"	"	"							
D	1	(12)	3	4	5	6	7	8	9	10	11	12
E	"											
F	"											
G	"											
H	"											

Transparent plate

A	1 Box 100 µL
B	"
C	"
D	"

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8.6.17

* Inoculating device colonies

Inoculate a colony in 5ml, 25 µg/mL Chloramphenicol LB

↓

Cam stock 35 mg/mL

So $5mL \times 25 \times 10^{-3} \frac{mg}{mL} = x\,mL \times 35\ mg/mL$

$x = \frac{25}{7}$

$= 3.57$ µl of 35 mg/mL Cam

For 100 mL LB ⇒ $100 \times 25 \times 10^{-3}$

$= x \times 35\ mg/mL$

$x = 3.57 \times 20$

$= 71.4$ µl of 35 mg/mL Cam

For 40mL ⇒ 8×3.57

$= 28.56$ µl

≈ 28.6 µl of 35 mg/mL Cam

↓

Take 5mL in 15mL falcon & pick a colony into it using 1mL tip

↓

Incubate at 37°C, 220 rpm.
16-18 Hrs
(Incubated @ 11.20pm)

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9/6/17

- First expt failed as Falcons opened inside the incubator.

12/6/17

- Inoculation for 2nd time

Results in Jyothish's nbk + iGEM google drive folder.

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15/6/17

Measurement track : Fluorescein

* 10× PBS (100 mL)
  - → 1.37 M NaCl - 8 gm
  - → 27 mM KCl - 0.2 gm
  - → 43 mM $Na_2HPO_4 \cdot 7H_2O$ - 1.15 gm
  - → 14 mM KH_2PO_4 - 0.2 gm

  } set pH to 7.5

* Diluting to 1× PBS (40 mL)
  - → Took 4 mL 10× PBS
  - → Added 36 mL distilled water
  - → Mix thoroughly

* Preparing Fluorescein standard
  Spin down the tubes 3000 rpm 30 sec
  ↓
  Resuspend in 1 mL 1× PBS
  (2× Fluorescein)
  ↓
  Take 500 µL & dilute with 500 µl
  of 1× PBS (1× Fluorescein)
  50 mM
  ↓
  Pipet 100 µl into the wells

Black plate used:

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Preparing our own 100 mM Fluorescein 1 mL

~~sodium~~

Molar mass of Fluorescein = 376.275 g

$$\text{Wt required} = \frac{\text{molar mass} \times \text{conc.} \times \text{vol}}{1000}$$

$$= \frac{376.275 \times 100 \times 10^{-3} \times 1}{}$$

$$= 0.0376275$$

$$\approx 0.03763 \text{ g}$$

Dissolve in ~1mL 1X PBS.

Wt of ~~sodiu~~ Fl. = 332.1

5mM Fluorescein solution = 10 mL

$$\text{Wt} = \frac{332.1 \times 5 \times 10^{-3} \times 10}{1000}$$

$$\approx 17 \text{ g}$$

↓

Settings: Std GFP settings.

96 Flatbottom Corning halfwell Black.

→

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	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	Bla
B	1	2	3	4	5	6	7	8	9	10	11	Bla
C	1	2	3	4	5	6	7	8	9	10	11	Bla
D	1	2	3	4	5	6	7	8	9	10	11	Bla

1 = 50 µM (1X sol^n)

serially 11 dilutions 1:1 500µl of PBS

Blank: PBS 1X 500 µl

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28/6/17

Transformation of GFP with ssRA tag.

Part : BBa_K1399008
Kit plate 5 7P pSB1C3 backbone.

Materials :
- Resuspended DNA to be transformed
- 10pg/μl Positive transformation control DNA (RFP on high-copy CAM resistant plasmid)
- Competent cells (50μl per sample)
- 1.5mL Microtubes
- LB medium (950μL per sample)
- Petri plates w/ LB agar + antibiotic (2 per sample)

Resuspend DNA in selected wells in the dist kit with 10μl dH_2O. Pipet up and down several times. let sit for few mins. Resuspension will be red with cresol red dye

↓

Label 1.5ml tubes with part name or well location. Fill lab bucket with ice & pre-chill 1.5ml tubes (one tube per transformation + 1 for control)

↓

Thaw competent cells on ice. This may take 10-15 mins for 260μl stock. Dispose off unused cells

↓

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Pipette 50µl of comp cells into 1.5ml tube + CONTROL

↓

Pipette 1µl ~~DNA~~ of resuspended DNA into 1.5ml tube. Gently pipette up & down a few times. Keep all tubes on ice

↓

Close 1.5ml tubes; incubate on ice for 30 min (Gently Flick / mix on ice)

↓

Heat shock tubes at 42°C for ~~45sec~~ 60sec

↓

Incubate on ice for 5min

↓

Pipette 950µl ~~SOC~~ ~~of~~ LB media to each transformation

↓

Incubate at 37°C for ~~1hr~~ 2 hrs at 220 rpm

↓

Pipette 100µl of each transformation into a petri plate & spread.

↓

Spin down cells at 6800g for 3mins & discard 800µl of supernatant. Resuspend the remaining cells in 100µl & pipette each transformation into the petri plate

↓

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Incubate overnight (14-18 hr) at 37°C

↓

Pick single colonies

Do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures & miniprep

↓

Count colonies for control transformation (100µl plate)

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28/6/17

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Plasmid constructs for Hijack Module

To be sequenced :-

① RBS + araC gene with ssrA + double terminator

RBS : BBa_B0030

AraC : BBa_C0080

ssrA : BBa_M0052

Double terminator : BBa_B0014

② DNA-A dependent promoter (Pol. A) + RBS

Pol. A promoter : BBa_K847210 (Add prefix)

RBS : BBa_B0030

③ RBS + LacI with ssrA + double terminator

RBS : BBa_B0032

LacI : BBa_C0012

ssrA : BBa_M0052

Double terminator : BBa_B0014.

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29/06/17 - 30/06/17

Interlab study (Repeat)

1st innoculation

2ml LB to 15 ml Falcons (X16)

↓

Add 1.42 µl of CAM to each

↓

Label (+ve, -ve, TD1-TD6 cultures 1&2)

↓

Wrap with alu. Foil

↓

Pick colonies using 200µl tips
(2 per device)

↓

Inocullated in 2ml LB + CAM

↓

Overnight incubation 37°C 250 rpm

2nd Inoculation.

50ml Falcons (X16) & label

↓

Add 8mL LB + CM

↓

Remove 40 µl LB From each

↓

Add 40 µl culture

↓

Wrap in Foil.

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~~At~~ 11 am 0 hr time pt (1.5 ml / eppy)

↓

Ice → 4°C

↓

Put Falcons back in incubator.

↓

1 pm : 2 hrs 3 pm : 4 hrs 5 pm 6 hrs time points ~~eluted in~~ aliquoted in eppys & kept at 4°C on ice.

↓

Plate reader measurement For fluorescence.

Black plate

Corning 3686, 96 well plate, half area, Flat bottom

P-1

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ve	-ve	TD1	TD2	TD3	TD4	TD5	TD6	Bl.	+ve	-ve	TD1
B												
C	→ Colony 1											
D												
E												
F	Colony 2 →											
G												
H												

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Miniprep Plasmid extraction.

Extraction of plasmid From GFP colonies (BBa_K139908) pSB1C3 backbone.

~~Transformation F~~

Inoculation of transformed colony for miniprep.

Take 2mL LB-CAM (25 µg/mL) in a 15 mL Falcon

↓

Pick one GFP transformed colony into the Falcon with 10µL tip

↓

Incubate at 37°C 220 rpm For 18 hrs

Miniprep plasmid extraction.

Pipette 1mL culture into 2 ~~1.5mL~~ 1.5mL eppys each

↓

Centrifuge at 8000 rpm (6800 rcF) For 3mins at 25°C

↓ → Discard supernatant.

Resuspend pellet in 125 µl P1 buffer

↓ → Add RNAse to P1 buffer, Write opened date

Pool them together into a 1.5mL eppy

↓ (lysis)

Add 250 µl P2 buffer & mix well by

inverting the tubes 4 to 6 times — NOT more than 5 mins

↓

P2 buffer: Put date on the bottle when opened → ~~NOT mo~~

↓

Add 350 µl N_3 buffer & mix by inverting the tubes 4 to 6 times

↓ 13,000 rpm

centrifuge at 17900 rcf at 25°C for 10 mins

↓

discard pellet

↓

Pipette 800 µl supernatant into the column

↓ 13,000 rpm

Centrifuge @ 17900 rcf for 60 sec

↔ Discard Flow through

Add 750 µl PE buffer to the bed

↓ 13,000 rpm

Centrifuge at 17900 rcf for 60 sec

↔ Discard Flow through

Centrifuge at 17900 rcf for 60 sec 13,000 rpm.

↓

Transfer the column into 1.5 mL eppy

↓

Temp: 37°C Add 50 µl EB & let is stand for 1 min ▷ can be heated to ~55°C before adding

↓

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Centrifuge at 17900 rcf for 60 sec
↓
store at -20°C labelled as
BBa_K139908, GFP, 5_7P

① P1 buffer: Resuspension buffer
(Tris-EDTA & RNase A)
Store at -4°C after adding RNase A
↳ Resuspension of bacteria in a suitable environment. EDTA binds to divalent ions (Mg^{2+}, etc) to disable the nuclease to degrade DNA.

② P2 buffer: Lysis buffer
(200mM NaOH & SDS) } high pH
base ↵ ↳ soap.
Destroys cell membrane → SDS
NaOH denatures DNA.

③ N3 buffer: Neutralization buffer
guanidine hydrochloride + acetic acid.
Renatures DNA: neutral pH
Precipitates as -SDS salt.

④ PE (?): useful to wash out other components
(Has buffer + salt + ethanol)
Prevents DNA from dissociating from column while you wash away other contaminants.

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(5) PB buffer: Has high salt content.
DNA binds more effectively to the Si-based matrix (?)

(6) EB : Contains 10mM tris at pH 8-9
↳ DNA is soluble in tris buffer.
('Usable' again)

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TAE buffer

50X Stock solution.
(Gel running buffer)

100ml solution :- 50X.

Add:

24.2g of Tris base,
5.71 ml of glacial acetic acid
10 ml of 0.5M EDTA (pH 8.0)
Make vol. upto 100ml with distilled water

To make 0.5M EDTA.
Molecular weight of anhydrous EDTA = 292.24 g/mol.

To make 20ml of 0.5M EDTA,

$$1000 : \frac{292.24}{2}$$

$$20 : x$$

$$x = \frac{20 \times 292.24}{1000 \times 2}$$

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0.5M EDTA.

We have Na_2 EDTA

Mol. wt 372.24 g

Total volume : 100 ml

∴ 18.612 g EDTA

dissolve in 80 ml

Add NaOH → it will dissolve when pH is correct (≈ 0.8)

Make volume upto 100 ml

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5/7/14 DNA loading Buffer (6X)

30% (V/v) glycerol } Total vol.
0.25% (W/v) bromophenol } 20 ml

6ml glycerol
50 mg bromophenol blue
14ml milli-q H_2O

RBS : 42.2 ng/μL 260 : 0.844
260:280 : 1.89 280 : 0445

GFP : 54.8 ng/μL 260/280 : 1.88

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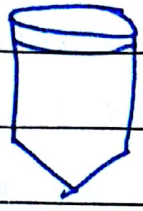
2/8/17

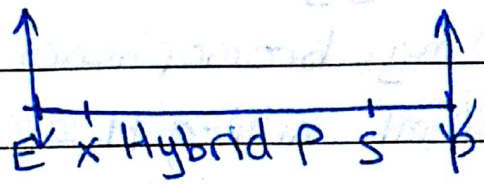
Hybrid

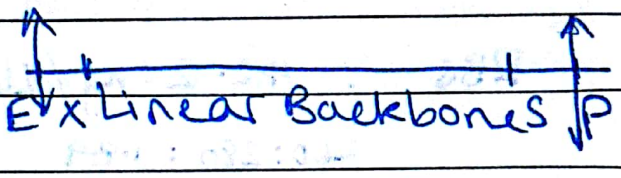
2A assembly: Hijack promoter & Linear backbone : pSB1C3

Digestion: pSB1C3 backbone

Backbone digest







~~Conc: 25 ng/µl~~ .

Conc: 25 ng/µl

1µl Backbone

3µl dH_2O

4µl Mastermix

Backbone

Enzyme mastermix For backbone

(25µl total ; 5 rxn)

5 µl NEB Buffer ~~3~~ 3.1 for ECORI

~~0.5 µl BSA~~

0.5 µl EcoR1 - HF

0.5 µl Pst1

0.5 µl Dpn1 (To digest DNA template from production)

18.5µl dH_2O

25µl

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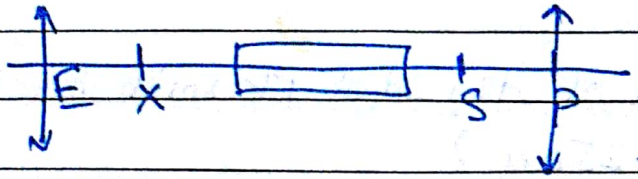
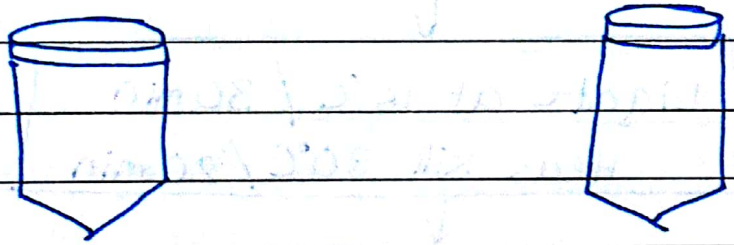
$\frac{39.3\,ng}{1\,\mu l} = \frac{25\,ng}{x\,\mu l}$ $x =$

Enzyme mastermix for part



Total 25 µl For 5 rxn.	2 rxn
5 µl NEB Buffer 3.1	2 µl
~~0.5 µl BSA~~	~~0.2 µl~~
0.5 µl EcoRI-HF	0.2 µl
0.5 µl PstI	0.2 µl
~~5~~ µl dH_2O	7.6 µl
25 µl	10 µl

Digestion



Backbone	Part
~~4 µl~~ ~~50 ng~~ 25 ng/µl	Conc = 39.3 ng/µl
25 ng backbone = 1 µl	25 ng part = 0.64 µl
Mastermix = 4 µl	Mastermix = 4 µl
dH_2O = 3 µl	dH_2O = 3.36 µl
8 µl	8 µl

↓

Digest each at 37°C / 30 min
Heat kill at 80°C / 20 min.
↳ Denaturation of enzyme

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$\frac{1}{8}$ = 25 ng Total = 25 ng in 8 µl
6.25 ng / 2 µl

Ligation

Add 2 µl of digested Plasmid Backbone
(= 6.25 ng)
↓
Take equimolar mount of part
(= ~~1 conc ng = x µl~~ / 4) = 2 µl
↓
Add 1 µl T4 DNA ligase buffer
↓
Add 0.5 µl T4 DNA ligase
↓
Add water upto 10 µl
= 4.5 µl
↓
Ligate at 16°C / 30 min
Heat kill 80°C / 20 min
↓
Transform with 1-2 µl of product.

→ check whether it contains ATP.

Reaction:		Control
Digested backbone	= 2 µl	2 µl
Part	= 2 µl	X
T4 DNA ligase buffer	= 1 µl	1 µl
T4 DNA ligase	= 0.5 µl	0.5 µl
water	= 4.5 µl	6.5 µl.
	10 µl	10 µl

Transformation result: No colonies in control & test after ~~24~~ 18 hours.

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4/8/17

Comp cells

2ndary ↓

Innoculate 50ml SOB with 200µl seed stock

↓

18 hrs incubation 220rpm 20°C
OD = ~~0.3~~ (~~0~~ 0.168)

↓

Transfer to 50ml Falcons kept on ice

↓

Centrifuge at 3000g at 4°C for 10 min

↓

Decant supernatant

↓

Resuspend in 16ml CCMB80 on ice
(Resuspend in 1ml first & then put 15ml)

↓

Incubate on ice for 20 min

↓

Centrifuge 3000g/ 4°C

↓

Decant supernatant

↓

Resuspend in 2ml CCMB80

↓

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OD measurement:

50 µl cell + 950 µl SOB

Blank : 50 µl ~~of~~ CMB80 + 950 µl SOB

↓

Measured OD =

OD of suspension = 0.073 × 20 × (Df)

≡ 1.460

↓

Dilute the cell suspension so that

Final OD = 1 to 1.5

(OD of suspension × Df ∈ (1 ; 1.5))

Dilution = x

Final OD = 1.46

↓

Aliquot 50 µl in 1.5 ml eppys

↓

* Incubate on ice for 20 mins

↓

x [Flash freeze]

↓

Store in -80°C in cryo box

(Label :

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EtBr gel.

1% gel

50 ml gel

OR 0.5g Agarose

↓

50 mL 1X TAE

↓

Microwave: 1.50 min + 10 sec

↓

Cool

↓

~5µl EtBr

↓

Pour

↓

Add comb

↓

Run at 80V For 1 hr.

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pSB1C3 backbone
mastermix (2X = 2rxn)
10µl total

NEB buffer 3.1	2 µl
~~0.2 µl~~	
EcoR1-HF	0.2 µl
Pst1	0.2 µl
DpN1	0.2 µl
dH_2O	7.4 µl
	10 µl

Hybrid promoter mastermix
(2X = 2rxn)
10µl total

NEB buffer 3.1	2 µl
~~Eco~~R1-HF	0.2 µl
Pst 1	0.2 µl
~~DpN1~~	~~0.2 µl~~
dH_2O	7.6 µl
	10 µl

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Digestion reaction.

psB1C3 backbone
~~Conc. 25 µg/ml~~
Conc = 25 ng/µl
25 ng backbone = 1 µl
Mastermix = 4 µl
dH_2O = 3 µl
8 µl

Part
Hybrid promoter
Conc = 25 ng/µl
Part = 1 µl
Mastermix = 4 µl
dH_2O = 3 µl
8 µl

↓

Digest at 37°C / 30 min
Heat kill at 80°C / 20 min.

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5c

11/8/17 QIAquick Gel extraction kit protocol

Excise the DNA Fragment From agarose gel w/ clean scalpel

↓

Weigh the gel slice in a colourless tube (= x mg)

↓

Add 3 vol. of buffer QG to 1 vol. of gel (= 3x μl)

QG buffer: has tris-EDTA For ↓ DNA damage

↓

Incubate at 50°C For 10 min / until completely dissolved.

Vortex every 2-3 mins

↓

Colour of gel should be yellow (pH ≤ 7.5). Otherwise, if orange, add 10μl 3M sodiumacetate

↓

Add 1 gel vol. of isopropanol (x μl) & mix

↓

Place a QIAquick spin column in a provided 2ml collection tube.

↓

Apply sample to QIAquick column & centrifuge For 1 min 13,000 rpm

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↓ → Discard Flowthrough

Discard Flow-through & place QIAquick column & ~~centrifuge~~ 1 min 13000 rpm
~~(Removes aggrose)~~

↓

~~To wash, add 0.75 ml PE Buffer & centrifuge 1 min 13,000 rpm~~

↓ → ~~Discard Flowthrough~~

↓

Add 0.5 ml of buffer QG to QIAquick column & centrifuge 1 min / 13,000 rpm

↓

To wash, add 0.75 ml PE buffer & centrifuge 1 min / 13,000 rpm

↓ → Discard Flow through

Discard Flowthrough & centrifuge the QIAquick column for an additional 1 min / 13,000 rpm

↓

Place QIAquick column into a clean 1.5 ml microcentrifuge tube

↓

To elute DNA add 50 µl of EB buffer / water to the center of the QIAquick membrane & centrifuge / 1 min / 13,000 rpm
(or 30 µl EB + 1 min incubation + centrifugat)

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↓

For gel analysis, add 1 vol. of Loading dye to 5 vol. of purified DNA.

Results: No bands on the gel
(1% gel 80 mV 2 log ladder)

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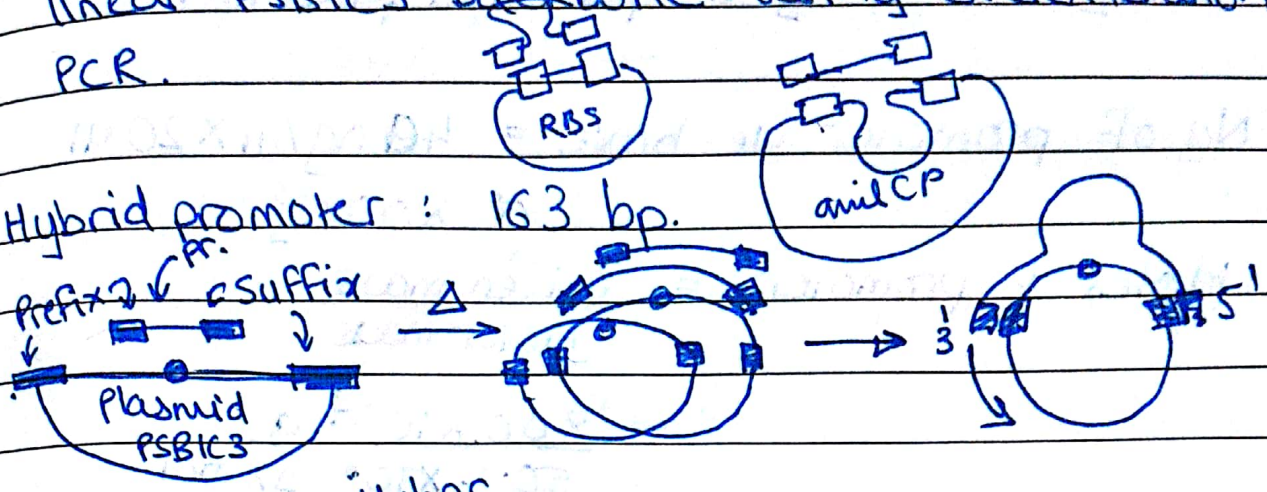
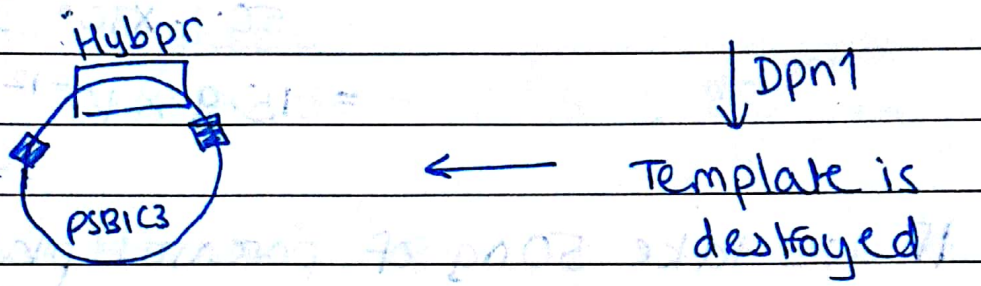
Overextension PCR

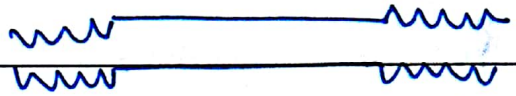
Aim: To ~~put~~ clone Hybrid promoter into the linear PSB1C3 backbone using overextension PCR.





Final product

RF cloning protocol



Imp: Conc. of Megaprimer : Conc. of plasmid :: 2:1 3:1 5:1 depending on concentration

~~Molar~~ mass of promoter: 50398.76 Da
$= 8.37 \times 10^{-20}$ g $\times A_0$

~~Molar~~ mass of Amil CP : 846385.85 Da
$= 1.4 \times 10^{-18}$ g $\times A_0$

Molar mass of promoter = 50.4×10^3 g/mol

Molar mass of Amil. CP = 8.43×10^5 g/mol

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62.6 1
50 ?

Secondary PCR optimal conditions.
A molar ratio of insert: plasmid ≥ 20
using 20-50ng of parental plasmid.

Ng of promoter we have = 40 ng/μl × 20 μl
≈ 800 ng

Moles of promoter = $\frac{\text{Given mass}}{\text{Molar mass}}$

$= \frac{800 \times 10^{-9}\ g}{50.4 \times 10^{3}\ g/mol}$

$= 15.9 \times 10^{-12}$ mol

If we take 50ng of parental plasmid,
Vol. taken =:
Conc of AmilCP plasmid = 62.6 ng/μl
Vol. taken = ?

62.6 1
50 ?

$? = \frac{50}{62.6} = 0.8\ \mu l$

Moles of parental plasmid = $\frac{50 \times 10^{-9}\ g}{8.43 \times 10^{5}\ g}$

≈ 6×10^{-14} mol

∴ We need to take $20 \times 6 \times 10^{-14}$ mol. of insert
$= 120 \times 10^{-14}$ mol
$= 0.012 \times 10^{-12}$ mol which we ha

50.4×10^{3} g/mol × 120×10^{-8} mol
$= 6048 \times 10^{-5}$ g = 0.6 mg

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~~6.048×10^{-5} g~~

~~0.6 ng of insert = ? volume~~

~~Conc = 40 ng / 1 µl~~

40×10^{-9} g ~~/ µl~~ ——— 1 µl

6.048×10^{-5} ——— ?

$$? = \frac{6.048 \times 10^{-4}}{40}$$

$$= 1.2 \times 10^{-12} \text{ mol}$$

Weight of insert =

1.2×10^{-12} mol × 50.4×10^{3} g/mol } Mol. mass of insert

$= 60.48 \times 10^{3-9}$ g

$\approx$ 60 ng

Vol. ~~60 ng~~ to be taken $\frac{60 \text{ ng}}{40 \text{ ng/µl}} = 1.5$ µl

100 ng plasmid = 1.6 µl

20X molarity of insert = 3 µl of insert.

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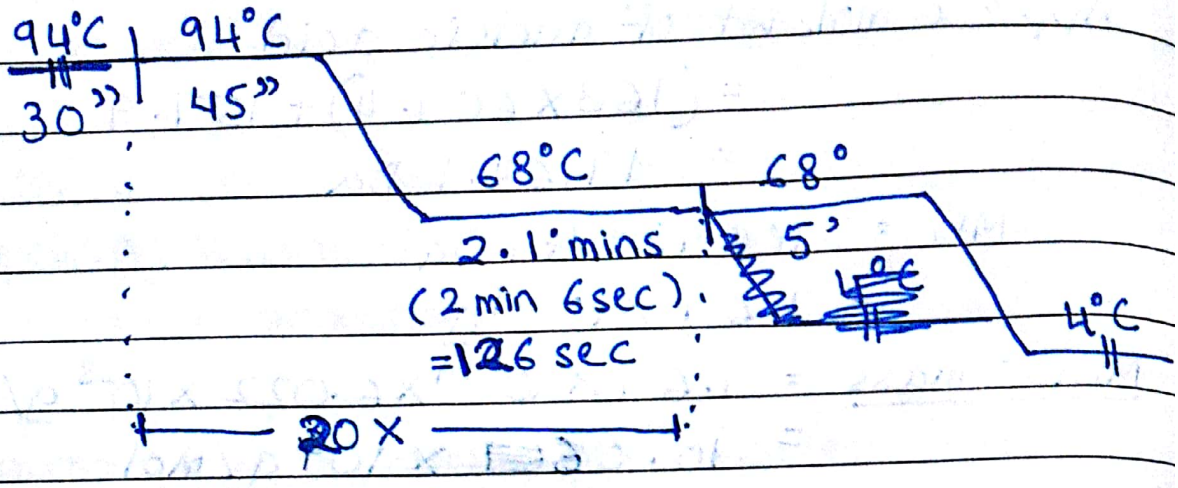
3 µL in 100 µL = 0.3 µl
0.3 µl 10

2-step PCR Tm = 72°C



↓

20 units of Dpn1

↓

2 hrs 37°C

↓

80°C /20 min inactivation

21/8/17 PCR

	pCAT3	RBS	Final conc.
10 X Pfx Buffer	1 µl	1 µl	1 X
10 mM dNTP	0.3 µl	0.3 µl	0.3 mM
50 mM $MgSO_4$	0.2 µl	0.2 µl	1 mM
Megaprimer	4 µl	4 µl	
Template DNA	2 µl	2 µl	
Pfx polymerase	0.4 µl	0.4 µl	1U
Distilled water	2.1 µl	2.1 µl	
	10 µl	10 µl	

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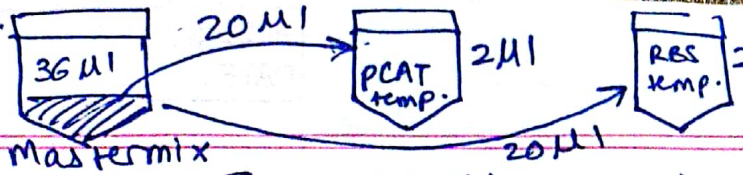

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Mastermix for 10 µl reactions: 2

10X PFx Buffer	2 µl
10mM dNTP	0.6 µl
50mM $MgSO_4$	0.4 µl
Megaprimer	8 µl
Template DNA	— $\frac{1}{2}$ µl each
PFx Polymerase	0.8 µl
Distilled water	4.2 µl
	20 µl

For 20 µl rxn

Mastermix

10X PFx Buffer	4 µl
10 mM dNTP	1.2 µl
50mM $MgSO_4$	0.8 µl
Megaprimer	8 µl
Template DNA	— 2 µl each
PFx Polymerase	1.6 µl
Distilled water	20.4 µl
	40 µl

Final optimized PCR (PFx platinum)

5'	15"		
94°C	94°C	1 min per kb	
		2 min 6 sec	
		68°C	4°C
	25X		

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↓

Thaw comp cells on ice
(50 µl MG1655, 50 µl DH5α) X2

↓

Pipette 1 µl of ~~cells~~ PCR product

↓

Close tubes. Incubate on ice for
30 min

↓

Heat shock at 42°C / 1 min

↓

Incubate on ice for 5 mins

↓

+ 250 µl SOC to each transformation
↳ 2 hrs at 37°C incubation

↓

Centrifuge at 8000g for 5 min

↓

Resuspend in 100 µl
~~↳ 2 hrs at 37°C incubation~~

↓

Plate on antibiotic plates

↓

Incubate at 37°C / 14 hrs

Results (1) RBS pSB1A3 backbone template
→ 3 colonies (MG1655)
(2) pCAT pSB1C3 backbone template
→ 3 colonies (DH5α)

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Antibiotic stocks

1. Ampicillin
   Stock : 100 mg/ml in 50% ethanol
   Working : 100 µg/ml
   Colour code : orange

2. Chloramphenicol
   Stock : 35 mg/ml in 100% ethanol
   Working : 35 µg/ml
   Colour code : Green

3. Kanamycin
   Stock : 35 mg/ml in distilled water
   Working : 35 µg/ml
   Colour code : Red
   Note : Stocks are stored in 1ml aliquots.

4. Tetracyclin
   Stock : 15 mg/ml in 50% ethanol
   Working : 15 µg/ml
   Colour code : Yellow
   Note : Light sensitive! Wrap in Aluminium Foil.

5 aliquots each : Weights taken :-

2. CAM 35 X 5 g = 175 g mg
3. Kan 35 X 5 g = 175 g mg
4. Tet 15 X 10 g = ~~75 g mg~~ 150 mg

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29/08/17

Miniprep of PCR product
(Hyb promoter in cam & Amp backbone)

Inoculation of transformed colonies

Take 2 ml LB-Antibiotic (LB-Cam + LB-Amp) (1 µl / ml) in a 15 ml falcol

↓

Pick one colony (From transformed plate) into the Falcon with 10 µl tip

↓

Incubate at 37°C / 220 rpm / 18 hrs

↓

Miniprep plasmid extraction

Time: 1 - 1½ hrs

Pipette 1 mL culture into 2 1.5 ml eppys each

↓

Centrifuge 8000 rpm (6800 rcf) for 3 min at 25°C

↓ → Discard supernatant

Resuspend pellet in 125 µl P1 buffer

↓

Pool them together in a 1.5 ml eppy

↓

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(Lysis)

↓

Add 250 µl P2 buffer & mix well by inverting the tube 4-6 times.

* DO NOT VORTEX
* Not more than 5 mins

↓

Add 350 µl N_3 buffer & mix by inverting the tubes 4-6 times

↓

Centrifuge at 13000 rpm (17900 rcF) at 25°C For 10 mins

↓ → Discard pellet

Pipette 800 µl supernatant into QIAprep column & centrifuge at 13,000 rpm / 60 secs

↓ → Discard Flow thr'

Wash QIAprep spin column by adding 0.5 ml Buffer PB & centrifuging For 30-60s.

Discard the flow-through.

↓

Centrifuge at 13,000 rpm For 60 secs

↓ → Discard Flow through

Add 750 µl PE buffer & centrifuge / 60s

↓

Centrifuge / 13000 rpm / 60 sec

↓ → Discard Flow through

Centrifuge / 13,000 rpm / 60 sec

↓ → Discard Flow thr'

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↓

Transfer column in 1.5 ml eppy

↓

Add 50µl Buffer EB at the center of each column.

↓

Stand For 5 min at 37°C

↓

Centrifuge at 13,000 rpm For 60sec

↓

Store at -20°C labelled Hyb_promoter_CAM or Hyb-promoter_AMP.

30/8/17

Hyb_pr_AMP conc: 93.6 ng/µl 260/280 = 2.01

Hyb_pr_CAM conc: 81.4 ng/µl 260/280 = 1.98

Strategy:

(1) Gel electrophoresis

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30/8/19

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Jyothish: Comp cell preparation; MG1655 Transformation efficiency test kit.

Take 50 µl of cells in 1.5 ml eppy

↓

Add 1 µl of 10pg/µl of RFP plasmid DNA from test kit.
Mix thoroughly

↓

30 min ice incubation

↓

1 min 42°C water bath

↓

5 min ice incubation

↓

Add 250 µl SOB & mix

↓

2.5 ~~2~~ hrs incubation at 37°C/ 220 rpm

↓

~~Res~~ Centrifuge at 3000 rpm/ 5 ± 2 mins

↓

Pellet resuspended in 150 µl

↓

Plate 150 µl on CAM plate

↓

Count colonies after 14 hrs incubation

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Colonies by extrapolation:-

= 533 x 4 = 2128 colonies.

Efficiency (cfu/µl) = 2.13×10^8 cfu/µl

1/9/17 Gel running for PCR product.

1.5g agarose in 1X TAE buffer

↓

Wells loaded:-

①	2 log ladder	5µl	loading dye
②	P-hybrid AMP	5µl	1 µl
③	RBS B0034 (Elowitz)	5µl	1 µl
④	P-hybrid CAM	"	"
⑤	pCAT promoter	"	"

↓

80V / 1 hr (8.30pm - 9.30pm)

↓

Result:- Same length bands

↓

Trouble shoot

↓

Did not digest the product

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4/9/17

Gel running PCR product.

Digestion

Restriction reactions

	pHyb amp	RBS Elowitz	pHyb cam	pCAT
RE: Xba1	1 µl	1 µl	1 µl	1 µl
10X Set cutsmart buffer	5 µl (1X)	5 µl (1X)	5 µl (1X)	5 µl (1X)
DNA	10.6 µl	20 µl	12.2 µl	21 µl
dH_2O	33.4 µl	24 µl	31.8 µl	23 µl
Total	50 µl	50 µl	50 µl	50 µl

1 Mg DNA calculations

Volume For 1 µg

pHyb amp : 93.6 ng/µl
pHyb cam : 81.4 ng/µl
RBS El : 50.1 ng/µl
pCAT : 47.8 ng/µl

93.6×10^{-3} µg — 1 µl
1 µg — ?

$? = \frac{1}{93.6} \times 10^3$

↓

1 hr incubation / 37°C

↓

Heat inactivation / 65°C 20 min.

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↓

1.5% agarose gel :-

↓

Take ∓ 0.75g agarose in 50ml TAE

↓

Heat & dissolve. ~~put comb~~

↓

Add ~ 1µl EtBr

↓

Pour into mould, insert comb

↓

Let it solidify for ~15 min

↓

Remove comb, put gel in the gel electrophoresis setup

↓

Wells :

1. 2log ladder 3µl
2. pHyb amp (dig) 5µl + 1µl loading dye
3. RBS elow. (dig) —
4. pHyb cam (dig) —
5. pCAT (dig) —

↓

80V, 1 hr

↓

Image gel under UV

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1.92% gel For some products.

↓

B ≥ 0.95g agarose in 50ml TAE

↓

Run at 80V For 2 hrs

1. 2 log ladder 3 µl
2. pHyb amp (dig) 5µl + 1µl LD
3. RBS elow ~~(dig)~~ ——
4. pHyb cam (dig) ——
5. pCAT (dig) ——

↓

get image:-

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Aarti's miniprep concentrations:-

	Conc. (ng/µl)	260/280
RBS elowitz (50µl)	66.6	1.94
pCAT (50µl)	63.7	1.89
Hyb pr Amp (25µl)	9	1.71
Hyb pr Cam (25µl)	112.8	1.9
RBS-Aarti (B0030) (50µl)	73.8	1.76

- Miniprep of Hyb. pr. Amp ⭐
- Digestion of Hyb pr. Amp & cam, RBS El., pCAT promotor with ~~Eo~~ EcoRI & Pst1 ⭐
- Running on 2% gel → agarose for confirmation. IF this fails ⭐

PCR overextension again with DpnI control

Ambiguous results → Colony PCR OR Assume it is the right construct & try to do 3A with GFP & RBS (Alongside 3A with G-block)

Miniprep ALL colonies separately & run on gel to see if there is ANY chance that we got the correct construct.

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- Transformation of PCR product(s) in ★ Jyothish's new cells
- 3A with GFP, RBS & backbone.

Miniprep of Hyb pr. Amp.

Inoculation :-

2ml LB + 200µl Amp, vortex

↓

1 colony 5:50pm

↓

18 hrs 220 rpm / 37°C

Plasmid miniprep Time: 1½ hrs

Pipette 1mL culture into 2 1.5ml eppys each

↓

Centrifuge 8000 rpm for 3min / 25°C

↓ Discard supernatant.

Resuspend pellet in 125µl P1 buffer

↓

Pool them together in a 1.5ml eppy

↓

Add 250µl P2 buffer & mix well by inverting 4-6 times

↓

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Add 350 µl N3 buffer
Mix by inverting 4-6 times
↓
Centrifuge 13000rpm / 25°C / 10 min
↓ → Discard pellet
Pipette 800 µl supernatant into QIAprep column & centrifuge at 13,000 rpm / 60sec
↓ → Discard Flow thr
Wash spin column by adding 1.5ml Buffer PB & centrifuging for 30-60 s
↓ → Discard Flow thr
Centrifuge at 13000 rpm / 60 s
↓ → Discard Flow thr
Add 750 µl PE buffer & centrifuge / 60 sec
↓ → Discard Flow thr
Centrifuge 13000 rpm / 60 s
↓ → Discard Flow thr
Transfer column into 1.5ml eppy
↓
Add 50 µl EB
↓

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Incubate at 37°C / 5 min

↓

Centrifuge at 13,000 rpm / 60 sec

↓

Store at −20°C labelled

pHybamp 8/9/17

Nanodrop results:-

4.5 ng/µl 260/280 = 1.63

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12/9/17 (14/9/17)

Digestion of miniprep products

pHyb AMP (old) ~~666 ng/µl~~	93.6 ng/µl
RBS elowitz	66.6 ng/µl
pHyb CAM	112.8 ng/µl
pCAT promoter	63.7 ng/µl

Enzyme efficiency table

Enzyme	Buffer 2.1	Buffer 3.1
✓EcoRI	100*	50
XbaI	100	75
SpeI	100	25
✓PstI	75	100

Double digestions:- 2 step.

Enzyme mastermix:- ~~4X reactions~~

	pHybamp	RBSEl.	pHybCAM	pCAT
DNA (100ng)	2.2µl	~~1.5~~ 3µl	1µl	~~3.2~~µl
NEB buffer 3.1	4µl	4µl	4µl	4µl
EcoRI	0.4µl	0.4µl	0.4µl	0.4µl
PstI	0.4µl	0.4µl	0.4µl	0.4µl
dH_2O	13.~~6~~µl	12.2µl	13.2µl	12µl
	20µl	20µl	20µl	20µl

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Digest at 37°C for 30 min

↓

Heat kill at 80°C / 20 min.

↓

Run on a 2% gel at
~~110V~~ 80V / 30 min.

~~13/9/17~~ ~~Transformation of plasmids.~~

Gel pic 14/9/17.

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13/9/17

Transformation of plasmids

Transformed 4 plasmids :-

1. Original PCR prod pHyb Amp
2. ——"—— pHyb CAM
3. First miniprep · pHyb Amp
4. ——"—— pHyb Cam-

Take 50µl competent cells

↓

1µl plasmid (s)

↓

25 min ice incubation

↓

1 min waterbath 42°C

↓

5 min ice incubation

↓

Add 250 µl SOB

↓

Shaker 2 hrs / 37°C / 220 rpm

↓

Pellet : Spin cells at 6000 rpm
For 7 mins

↓

Resuspend pellet in 100 µl SOB

↓

Plate

↓

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37°C / 18 hrs incubation

↓

Results.

Results

1. Original PCR prod pHyb Amp:-
   1 colony
2. Original PCR prod pHyb CAM:-
   Zero colonies
3. Miniprep Amp: Colonies of diff sizes.
4. Miniprep CAM : 7-8 colonies

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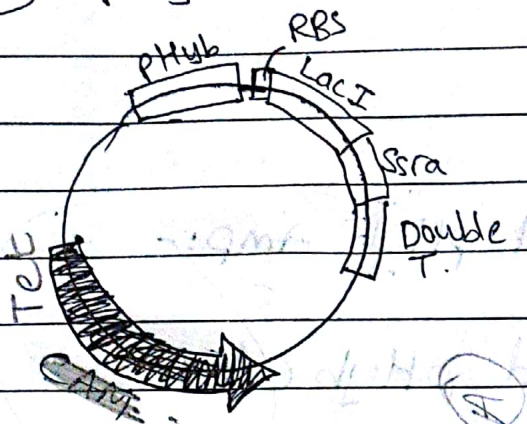
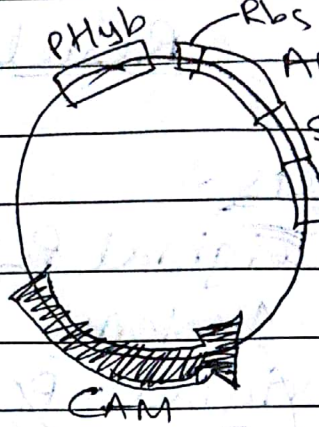
3A assembly

Goal : ① 3A of pHyb (g-block) + AraC (g-block) + CAM linear backbone (PSB1C3)

② pHyb + LacI (g-block) + ~~CAM~~ TET L·B (PSB1T3)





Digest

① Enzyme mastermix: For Plasmid Backbone (~~100~~ ng, 5 rxns) (25 µl)

	E & P		DNA 4 µl
(i)	~~5 µl~~ NEB Buffer 3.1	5 µl	1 µl
(ii)	EcoRI-HF	0.5 µl	0.2
(iii)	Pst 1	0.5 µl	0.2
(iv)	Dpn1	0.5 µl	0.2
(v)	dH_2O	18.5 µl	4.4
		25 µl	10 µl

② 4 µl of above mastermix + 4 µl (100ng) of plasmid backbone

4 hrs / 37°C
80°C / 20 min

Incubate 37°C / 30 min
Heat kill 80°C / 20 min

Set thermomixer h time!

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(2) Part A **pHyb - gblock** ★

Conc. by nanodrop measurement - 39.3 ng/µl

(i) SpeI + EcoRI — We cut smart

	E & S
NEB Buffer 2.1 (From lab)	2 µl
Spe I	0.2 µl
dH_2O	7.8 µl
	10 µl

↓

3 µl of master mix
1 µl of dH_2O
~~100~~ 150 ng pHyb = 4 µl

↓

↓

DNA	4 µl
Buffer	1 µl
E	0.2
S	0.2
dH_2O	4.6
	10 µl

Incubate 37°C / 30 min ↓ 4 hrs / 37°C
Heat kill 80°C / 20 min. ↓ 80°C / 20 min

Step-1 & step 2 (i) should be done together

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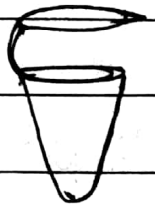
(ii) EcoRI digestion in buffer 3.1

(a) 8 µl digested product
(b) 0.8 µl buffer 2.1
(c) 0.8 µl Buffer converter 8 8
(d) ~~5.6~~ µl dH_2O 2.4
(e) 0.8 µl EcoRI 10.4 5.6
16 µl



Incubate 37°C / 30 min
Heat kill 80°C / 20 min.

Part B

Enzyme mastermix (gblock)

	rbsAraC	rbsLacI
~~10×~~ ~~5~~ NEB buffer 3.1	1 µl	1 µl
XbaI	0.2 µl ✓	0.2 µl
PstI	0.2 µl ✓	0.2 µl
dH_2O	8.6 µl	8.6 µl
	10 µl	10 µl

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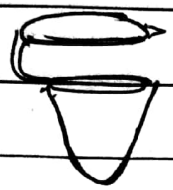
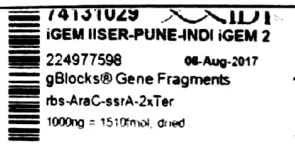
100 ng DNA (~~25~~ 4 µl)
+
4 µl mastermix
↓

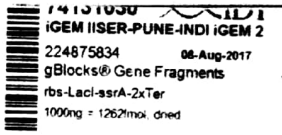


Incubate 37°C / 30 min
Heat kill 80°C / 20 min

This can be done in step 1.

gblocks





1000 ng
↓
added 40 µl
TE
↓
Final conc =
25 ng/µl

1000 ng
↓
Added 40 µl
TE
↓
Final conc =
25 ng/µl

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TE buffer

Recipe

1M Tris-Cl buffer	10ml	10^{-2} moles
0.5 M EDTA solution	2 ml	10^{-3} moles
distilled H_2O	988 ml	
	1L	

100ml

1M Tris Cl	1ml	10mM
0.5M EDTA	200µl	1mM
H_2O	98.8ml	
	100mL	

Ligation

Add 2 µl of digested plasmid backbone

↓

equimolar conc. of pHyb & g-block

pHyb: 4 µl

g-block ~ 2 µl

↓

1µl T4 DNA ligase buffer

↓

0.5µl T4 DNA ligase

↓

Add water upto 10 µl [0.5µl]

↓

Ligate at 16°C / 30min

Heat kill 80°C / 2 min

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Ligation mastermix : 4 reactions
T4 ligase buffer : 4 µl
T4 DNA ligase : 2 µl
dH_2O : 2 µl

3A ligation :- 2 µl M Mix + 4 µl promoter + 2 µl gblock + 2 µl backbone.

Transformation

50 µl comp cells + 1 µl ligated DNA
(LacI ligated, AraC ligated)
↓
30 min ice incubation
↓
42°C / 1 min waterbath
↓
5 min ice incubation
↓
Add 250 µl SOB; mix thoroughly
↓
150 rpm / 37°C for 2 hrs
↓
~~Plated / Incubate 37°C / 18 hrs~~
~~RE~~ Centrifuge 6000 rpm / 1 min
↓
Resuspend pellet in 100 µl SOB
↓
Plate + 37°C / 18 hrs incubation

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Results:- No colonies seen.

3/10/17

Troubleshooting #3A assembly

- Did another ligation following same protocol For LacI gblock + pHyb + $tetR^-$ backbone
- Transformation
- No colonies seen.

- Problem appears to be in digestion.

IDT website : 2A assembly of gblock

Digestion:

Product	gblock Frag
DNA	100ng
10X buffer	3µL
RE	1µl (each)
MQ H_2O	To 30µl

Ligation:

~~Li~~ Product	Amnt
Linearized vector	50ng
gblock	3-5X molar excess*
2X NEB ligase buf.	10µL
Ligase	1µl
dH_2O	upto 20µl

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Ratio vector: gblock = 1:3 (molar ratio)

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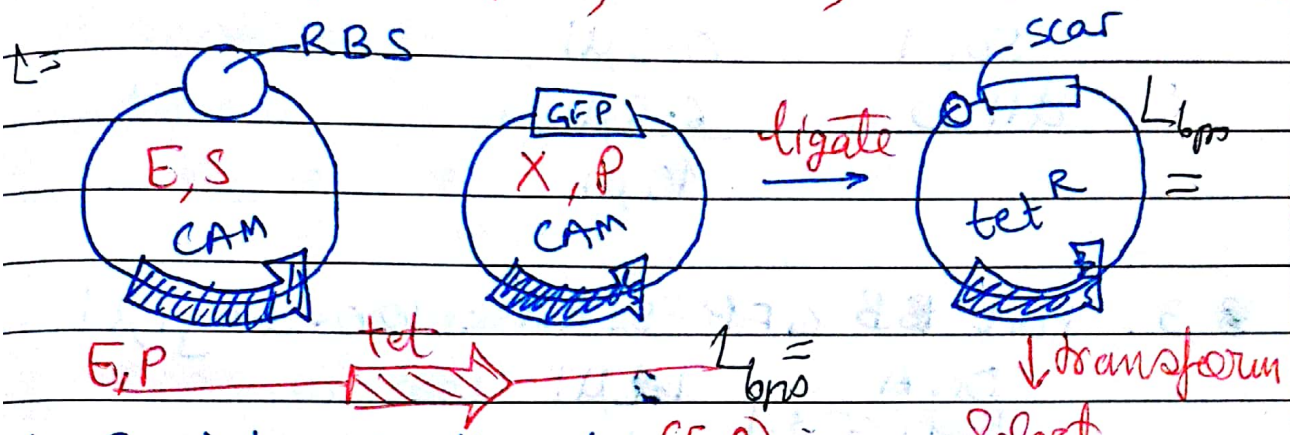
↓

Centrifuge briefly & incubate at RT for 5 min.

~~E1H033~~ / K139008

GFP-ssra & RBS B0030 in pSB1T3

E: EcoR1, S: Spe1, X: Xba1, P: Pst1



1. Backbone digest (E,P)

NEB buffer 3.1	1 µl
EcoRI HF	0.2 µl
Pst1	0.2 µl
Dpn1 (NEB)	0.2 µl
DNA	4 µl
dH_2O	4.4 µl
	10 µl

↓

4 hrs / 37°C

20 min / 80°C

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2. Part A RBS : 73.8 ng/µl
(140 ng)

DNA	2 µl
Cutsmart	1 µl
EcoR1	0.2 µl
Spe1	0.2 µl
dH_2O	6.6 µl
	10 µl

3. Part BB GFP-SSRA : 120.3 ng/µl

DNA	1 µl
Buffer 3.1 ~~Cutsmart~~	1 µl
X ~~EcoR1~~	0.2 µl
P	0.2 µl
dH_2O	7.6 µl
	10 µl

Molar wt of all 3 constructs:-
Molecular wt in daltons (ds DNA) = (# nucleotides × 607.4) + 157.4 Da
Source thermofischer
1 Da = 1.67×10^{-24} g
A_o = 6.023×10^{23} molecules/mol

① GFPssRA : 2823
Molar mass : 1724864.5 g/mol

② RBS : 2085
M.M : 1273984.5 g/mol

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③ pSB1T3 : 2461

MM 1503451.2 g/mol

1M = 1503451.2 g in 1L

~~$M_1C_1V_1 = M_2C_2V_2$~~

~~$1 \times 1L = M_2 \times C_4 \times \mu l$~~

$M_2 = \frac{1}{4 \times 10^6} M$

$M_2 = 0.25 \times 10^{-6} M$

Conc of backbone = 25 ng/µl

4 µl taken

= 100 ng

1M = 1503451.2 g

?M = 100×10^{-9} g

?M = $\frac{100 \times 10^{-9}}{1503451.2}$

?M = 6.65×10^{-14} M

② GFPssRA.

1M 1724864.5 g

6.65×10^{-14} ?

? = $6.65 \times 10^{-14} \times 1724864.5$

= 1.14×10^{-7} g

= 114 ng

≈ 1 µl

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RBS

1M 1273984.5

~~6.65 x 10^-14 g~~

6.65×10^{-14} ?

? = 0.084×10^{-7}

= 8.4 ng

= 1 µl

↓

37°C / 4hrs

20 min / 80°C

Ligation

2 µl digested backbone
2 µl Part A digest
2 µl Part B digest
1 µl T4 DNA ligase buffer
0.5 µl T4 DNA ligase
2.5 µl dH_2O
10 µl

↓ 16°C / overnight (Thermomixer cold)
↓ 80°C / 20 min ← Transform (MG1655) before heat-killing.
↓ root mach
↓ stored @ -20°C

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Transformation:

50µl comp cells + 1µl ligated product
↓
30 min ice incubation
↓
1 min 42°C water bath.
↓
5 min ice incubation
↓
250µl SOC* (instead of LB)
↓
150 rpm 37°C 2 hrs
↓
Plate on tet antibiotic LB plate

Control: 50µl comp. cells w/ plasmid on tet antibiotic plates

Results

	# colonies
Negative control:	0
Ligated product:	44

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Calculations for pHyb 3A w/ LACI & AraC

Molar mass of promoter: 50.4×10^3 g/mol

Molar mass of psB1T3 : 1503×10^3 g/mol

Molar mass of LacIgblock

bp: ~~2044~~ 1283 bp

MM 784004.3 g/mol

Molar mass of AraC gblock

bp: 1072

MM: 65509~~=~~4.3 g/mol

~~=~~ Conc. of psB1T3 : 25 ng/µl

4 µl : 100~~=~~ ng

= 6.65×10^{-14} M

For promoter:-

6.65×10^{-14} M = ?

1M : 50.4×10^3 g ~~mol~~

? = 335.16×10^{-11} ~~=~~ g

~~=~~ = 3.35 ng

~~=~~ ~ 0.1 µl ???

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~~Digestion~~

~~Port A : P.Hyb Conc : 39.3 ng/µl~~

~~1 µl = 39.3 ng~~

~~DNA 1 µl~~

~~2A assembly~~

DNA-A w/o SSRA 1466 bp : 25 ng/µl

MM: 895807.8 g/mol

Fts-Z w/o SSRA 1197 bp : 25 ng/µl

MM: 731462.8 g/mol

~~Conc~~ pSB1C3 : 2070 bp : 25 ng/µl

MM: 1264820.3 g/mol

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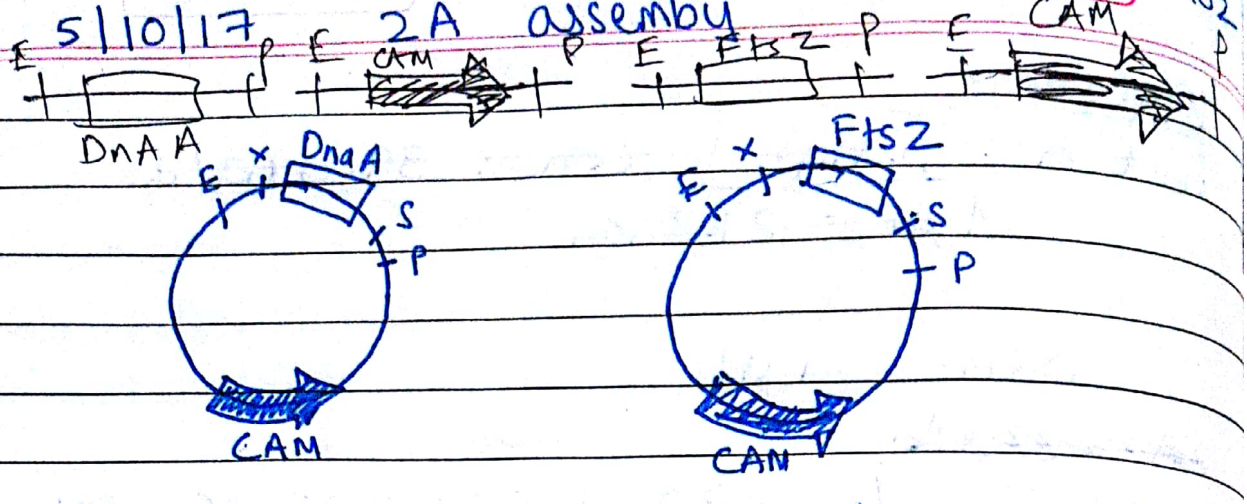
5/10/17 2A assemby



Backbone double digest

Linear backbone pSB1C3 with E & P

10 µl reaction.

DNA-A & Fts-Z with E&P also ↓ Same rxn

* Component ~~PSB1C3~~	10 µl reaction
DNA (DNA A & FtsZ)	4 µl
10 x buffer 3.1	1 µl
EcoRI	0.2 µl
PstI	0.2 µl
Nuclease free water	4.6 µl
	10 µl

↓

Incubate at 37°C for 4hrs

↓

Heat kill 80°C / 20 min.

for backbone *	
DNA	4 µl
10 x buffer 3.1	1 µl
EcoRI	0.2 µl
Pst I	0.2 µl
DpnI	0.2 µl
dH_2O	4.4 µl

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Ligation. (2A)

T4 DNA ligase should be added last

Molar ratio of 1:3 vector to insert reqd.

2 µl digested backbone

$2 \times \frac{42}{105}$ µl = actual bb DNA

0.8 µl = actual bb DNA

i.e. 25 ng/µl × 0.8 = 20 ng

1264820.3 g/mol = 1 M

20×10^{-9} g = ? M

$? M = \frac{20}{1264820.3} \times 10^{-9}$

$= 1.58 \times 10^{-14}$ M

Molarity of parts = 4.74×10^{-14} M.

g of DNA-A reqd:

895807.8 g — 1 M

? g — 4.74×10^{-14} M

$4246128.9 \times 10^{-14}$ g

= 42.4 ng ≈ 2 µl (~~conc = 25 ng/µl~~)

~~100 ng~~ × $\frac{2}{5}$ = ~~40 ng~~

$x \times \frac{2}{5} \times 25 = 42.4$ ~~ng~~

$x = 4.24$ µl.

Vol DNA-A = 4.24 µl.

≈ 4.2 µl

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g of Fts-Z reqd:

731462.8 g — 1 M

? — 4.74×10^{-14} M

$? = 34671133.6 \times 10^{-14}$ g

$= 34.6$ ng.

Vol. of Fts-Z ≈ 3.45 µl

Ligation

Component	DNA-A	Fts Z
10X T4 ligase buffer	1 µl	1 µl
Vector DNA digest	2 µl	2 µl
Port DNA digest	4.2 µl	3.5 µl
T4 DNA ligase	0.5 µl	0.5 µl
dH_2O	2.3 µl	3 µl
	10 µl	10 µl

↓

overnight 16°C / waterbath immersed completely

↓

Transform (MG1655)

↓

Heat inactivate 80°C / 20 min

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Plasmid isolation

of 3A : GFP-ssRA + RBS in CAM.

Picked 6 colonies & inoculated each in 2ml SOB overnight at 37°C / 150 rpm

Miniprep :

Pipette 1mL culture in 1.5ml eppy each. Label C1, C2, ..., C6

↓

(+) Centrifuge 8000 rpm / 3 min / 25°C

↓ → Discard supernatant

Resuspend in 125 µl P1 buffer

↓

Add 125µl P2 buffer & invert 6 times
Don't vortex NOT more than 5 min

↓

Add N3 buffer 175µl & invert 6 times

↓

Centrifuge at 13000 rpm / 25°C for 10 min

↓ → Discard pellet

Pipette → 400 µl supernatant into QIAprep column & centrifuge at 13000 rpm / 60 sec

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↓ Discard Flow thr

Centrifuge at 13,000rpm / 60sec / 25°C

↓ Discard Flow thr

Add ~~750µl~~ 375 µl PE buffer & centrifuge at 13000rpm / 60sec

↓ Discard Flow thr

centrifuge at 13000 rpm / 60sec

↓ Discard Flow thr

Transfer column in 1.5 ml eppy

↓

Add 50 µl EB at the centre of each column

↓

Stand for 5 min at 37°C

↓

Centrifuge / 13000 rpm / 60sec

↓

Store at -20°C

~~Labelled~~:

~~thg~~ ~~&~~

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Nanodrop:	conc. (µg/ml)	260:280
RBS-GFPssRA Colony1	986.5	1.97
RBS-GFPssRA Colony2	116.7	1.71
RBS-GFPssRA Colony3	109.3	1.83
RBS-GFPssRA Colony4	78.1	1.93
RBS-GFPssRA Colony5	107.4	0.94
RBS-GFPssRA Colony6	105.0	1.92

Digestion of miniprepped product + 6/10 gel running.

Digestion with Pst1

Buffer 3.1

Total vol 10 µl per rxn

Each rxn

1x Buffer 3.1	1 µl
DNA	2 µl
Enzyme (Pst1)	0.2 µl
dH_2O	6.8 µl
	10 µl

Mastermix (6 rxns) 30 µl

→ 6 µl NEB buffer 3.1

→ 0.6 µl Pst1

→ 23.4 µl dH_2O

① 4 µl MM + 2 µl C1/C2/C3/C5/C6 + 2 µl dH_2O

② 4 µl MM + 3 µl C4 + 1 µl dH_2O

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0.8% gel Single cut w/ PstI

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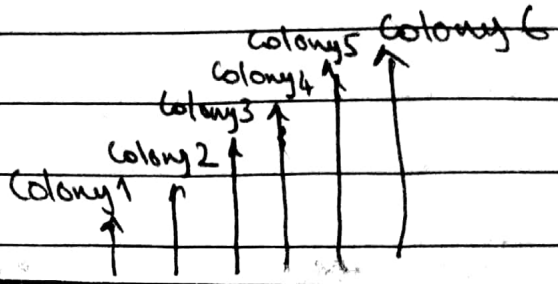

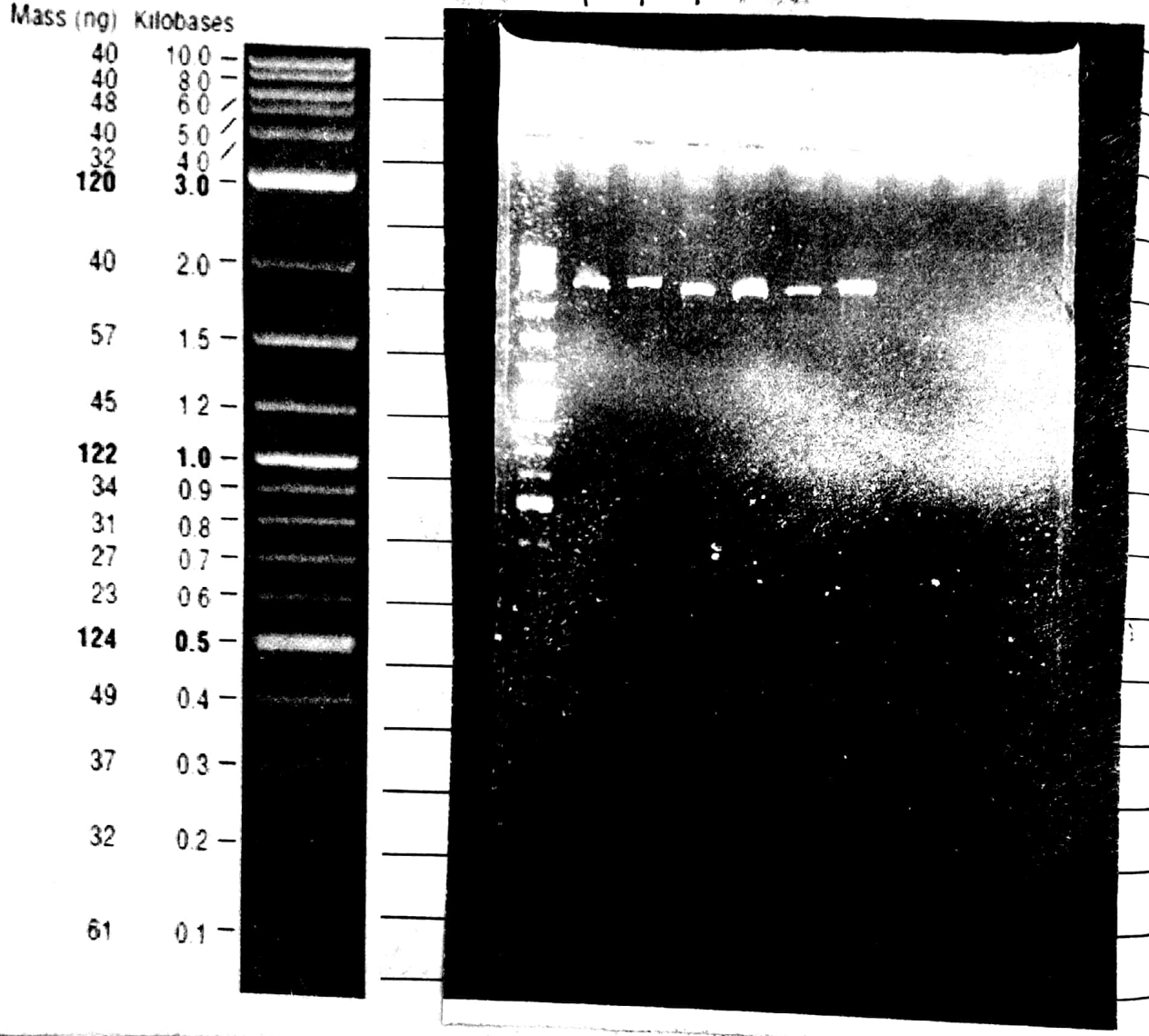

Expected size ~3.2Kb is seen.

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Plasmid isolation of FtsZ 2A

Inoculation :

5 colonies in 2ml, 220rpm, overnight at 37°C

Miniprep :

Pipette 1 ml culture into 1.5ml eppy each labelled C1, C2, C3, C4, C5 + 1 eppy for balance (distilled H_2O)

↓

↺ Centrifuge 8000 rpm / 3 min / 25°C

↓ → Discard supernatant

Resuspend in 125µl P1 buffer

↓

Add 125µl P2 buffer
Invert 6 times & mix

↓

Add 175µl N3 buffer
Invert 6 times & mix

↓

↺ Centrifuge 13000 rpm / ~~35~~°C / ~~10~~min

↓ → Discard pellet

Pipette → 400µl supernatant in Qiaprep column

↓

↺ Centrifuge at 13000 rpm / 60sec

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↓ → Discard Flowthr

↺ centrifuge at 13,000 rpm / 60sec / 25°C

↓ → Discard flow thr

Add 375 µl PE buffer

↓

↺ Centrifuge 13000 rpm / 60 sec / 25°C

↓ → Discard flow thr

↺ Centrifuge 13000 rpm / 60sec / 25°C

↓ → Discard Flow thr

Transfer column in 1.5ml eppy

↓

Add 50 µl EB & let stand / 5 min / 37°C

↓

Centrifuge 13000 rpm / 60sec / 25°C

↓

Store at -20°C

Nanodrop Measurements	Conc (µg/mL)	260:280
Colony 1	55.4	1.994
Colony 2	36.2	1.786
Colony 3	52.2	1.96
Colony 4	54.5	2.007
Colony 5	50.34	1.98

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Single cut plasmid (PstI) run on 0.8% gel

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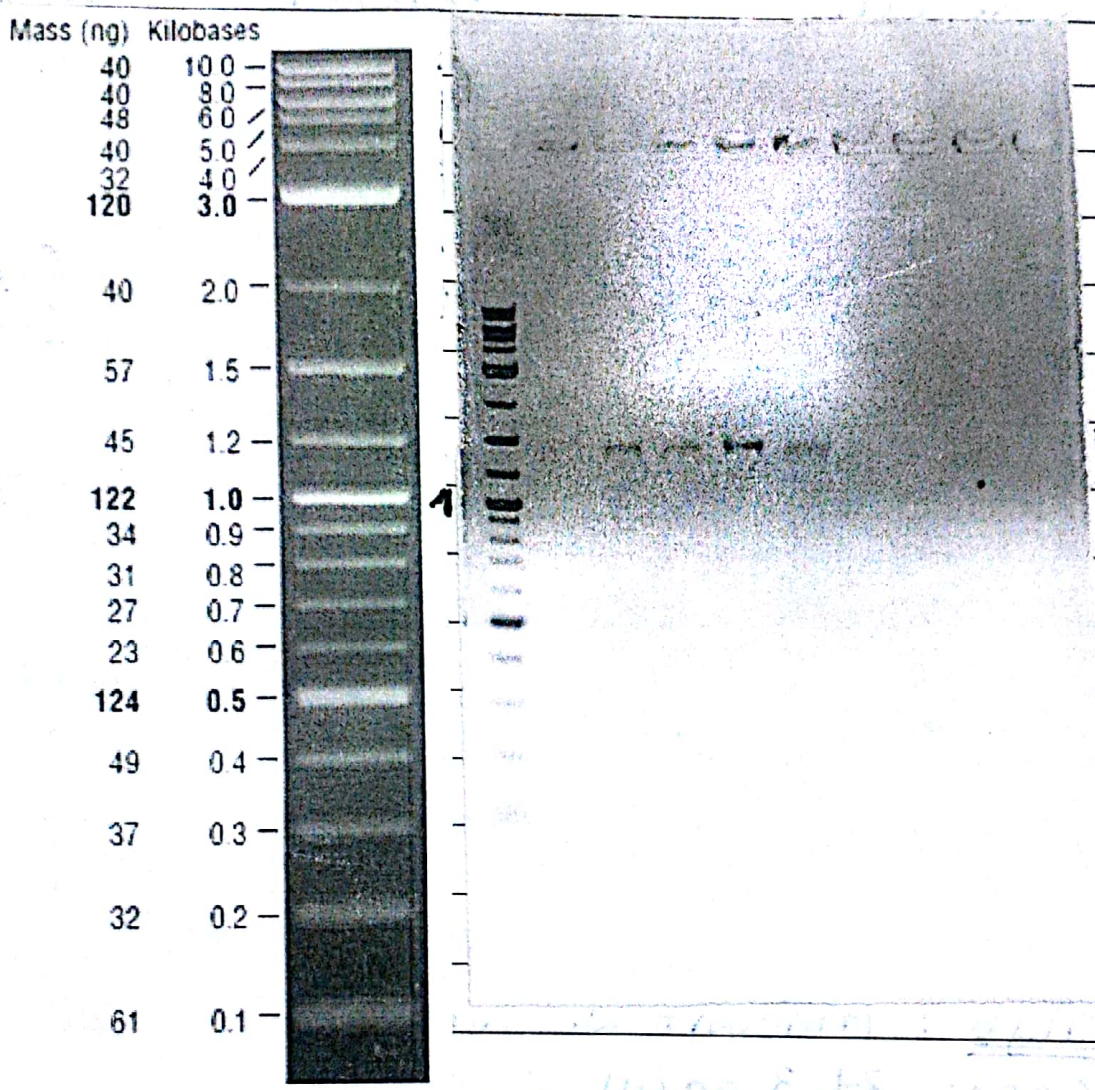
Gel



Expected size

2070 + 2070 = 4140 bp Bb-Bb ligation

1197 + 2070 ≈ 3.2kb Correct construct

1.5kb ??

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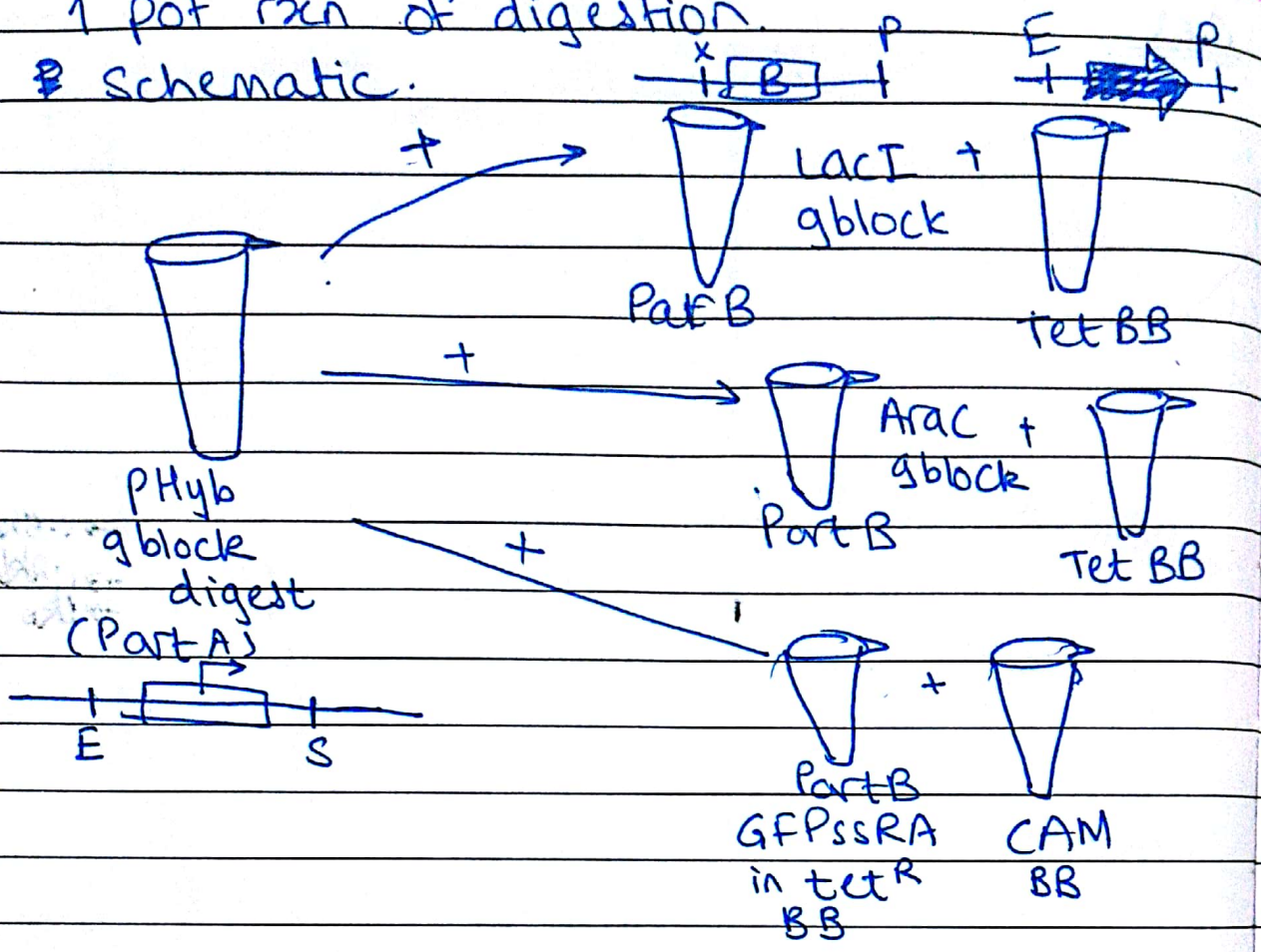
3A w/ pHyb

1 pot rxn of digestion.

Schematic.



Digestion of Part A

pHyb : Amount we have ~2 µl - 2.5 µl

Conc: 39.3 ng/µl ∴ ~ 80 ng - 100 ng.

Digestion rxn:

~~3 / 2 µl~~ pHyb	2/3 µl
EcoRI	0.2 µl
~~PstI~~	
SpeI	0.2 µl
10x Cutsmart	1 µl
dH_2O	6.6 / 5.6 µl
	10 µl

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$\mu g/ml = \frac{10^3}{10\mu l} = \frac{ng}{1\mu l}$

↓

Digest at 37°C For 3 hrs
Heat inactivation at ~~80~~ 80°C For 20 min

Part B : LacI, AraC, RBS-GFPssRA

Conc: LacI gblock 25 ng/µl
AraC gblock 25 ng/µl
RBS-GFPssRA (C1) 96.5 ng/µl

Digestion reaction

	LacI	AraC	RBSGFP
Part	4µl	4µl	2µl
XbaI	0.2µl	0.2µl	0.2µl
PstI	0.2µl	0.2µl	0.2µl
Buffer 3.1	1µl	1µl	1µl
dH_2O	4.6µl	4.6µl	6.6µl
	10µl	10µl	10µl

↓

Digest at 37°C For 3 hrs
Heat inactivation at 80°C For 20min.

Backbone Conc: 25ng/µl

	Tet^R	CAM^R	(10µl)
Part	4µl	4µl	
EcoRI	0.2µl	0.2µl	
PstI	0.2µl	0.2µl	
DpnI	0.2µl	0.2µl	
3.1 ~~DNA~~ Buffer	1µl	1µl	
dH_2O	4.4µl	4.4µl	

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↓

Digest at 37°C / 3hrs
Heat inactivat^n 80°C / 20 min

Ligation

Molar masses: BP

Tet backbone: 1503451.2 g/mol 2461
CAM backbone: 1264820.3 g/mol 2070
pHyb : 50.4×10^3 g/mol 163
LacI gblock : 8×10^5 g/mol 78.4004.3 g/mol 1283
AraC gblock : 655094.3 g/mol 1072
~~Lac~~ GFPssRA : 2092149.3 g/mol 3.2kb
21×10^5 g/mol

Ligation rxn
2 µl digested backbone

$= \frac{6.65 \times 10^{-14}}{1.58 \times 10^{-14}}$ M of pSB1T3
M of pSB1C3

g
50×10^3 1M
80-100ng ?

① For pHyb & GFPssRA.

~~pHyb~~ ~~$\frac{6.65 \times 1}{1.58 \times 10^{-14} \times 2}$~~ =

~ 80-100 ng = $1.6 - 2 \times 10^{-12}$ M

$1.58 \times 10^{-14} \times 2 = (1.6 - 2) \times 10^{-12} \times x$

$x = (1.975 - 1.58) \times 10^{-2}$ µl

$\therefore$ $x = 0.2$ µl ↓ 0.02 µl

can't measure

RBS
GFPssRA

193 ng = 9.1×10^{-14} M

$1.58 \times 10^{-14} \times 2 = 9.1 \times 10^{-14} \times x$

~~$x = 0.3044$~~

$x = 0.35\ \mu l \sim 0.4\ \mu l$

(2) ~~AM backbone~~ Tet backbone.

$2\ \mu l = 6.65 \times 10^{-14} M$

(a) LacI qblock ; pHyb

pHyb ~ 0.02 µl But can't measure
⇒ 0.2 µl

LacI

100 ng = ? M

$0.125 \times 10^{-12} M$

$= 1.25 \times 10^{-13} M$

$6.65 \times 10^{-14} \times 2 = x \times 1.25 \times 10^{-13}$

$x = 10.64 \times 10^{-1}$

$x = 1\ \mu l$

(b) AraC qblock

Comparable M.W

⇒ x ~ 1 µl

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Ligation rxn

	LacI (+et)	AraC (+et)	GFPssRA (CAM)
~~1X~~ T4 Ligase	0.25	0.25	0.25
10x buffer	1 µl	1 µl	1 µl
Vector digest	2 µl	2 µl	2 µl
Part Digest	1 µl	1 µl	0.4 µl
pHyb	0.2 µl	0.2 µl	0.2 µl
dH_2O	5.3 µl ~~5.6 µl~~	5.3 µl 5.6 µl	~~5.3 µl~~ 5.9 µl
	10 µl	10 µl	10 µl

↓

Ligate at 16°C / overnight
inactivat" 80°C / 20min

↓

Transformation.

~~↓~~

Religation of Dna-A

T4 ligase	0.5 µl
T4 ligase 10X buffer	1 µl
PSB1C3	2 µl (Digested) E, P
DnaA	4.2 µl (Digested) E, P
dH_2O	2.3 µl
	10 µl

Transformed all the above ligated products along with FtsZ + PSB1C3, Snehal's construct: Hyb Promoted +GFP + Pet15B (Amp)
Bb-Bb control (PSB1C3, E|P)

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No of colonies obtained
(i) AraC contruct - 0
(ii) LacI construct - 0
(iii) Hyb Promoter + RBS + GFPssRA(CAM) - 2
(iv) FtsZ - 0
(v) DnaA - 0
(vi) Snehal (GFP) - 129.

Miniprep of 2 colonies of (iii) 13/10/17

Inoculation: 2ml → LB, 1 colony, 2 Falcons. 220 rpm, 37°C, 18 hrs.

Miniprep

Pipette 1ml culture in 1.5ml eppy.
↺ Centrifuge 8,000 rpm, 25°C, 3 min
↘ Discard Flow thr.
Add 125 µl P1 buffer & resuspend
↓
Add 125 µl P2 buffer. Invert
↓
Add 175 µl N3 buffer. Invert
↓
Centrifuge 8.13×10^3 rpm, 10 min, 25°C
↘ Discard pellet
Pipette 400 µl into column
& Centrifuge @ 13000 rpm, 60 sec, 25°C

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↕ Disc. Flow thr'

↺ Centrifuge 13,000 rpm 25°C 60sec

↕ Disc. Flow thr'

Add 375 µl PE buffer &

↺ Centrifuge 13000 rpm 25°C 60sec

↕ Disc Flow thr'

~~Add~~ ↺ centrifuge 13000 rpm /60sec

↕ Disc Flow thr

Transfer into eppy

↓

Add 50 µl EB

↓

Let Stand @ 37°C / 5 min

↓

↺ Centrifuge 13000 rpm / 60 sec

↓

Store at -20°C

Nanodrop

		360/280	260/230
① Colony 1 :	62.770 µg/ml	1.411	1.83
② Colony 2 :	64.949 µg/ml	2.099	1.104

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20/10/17

Plan For Last Week.

① ~~B~~ 2 hrs inoculation of saturated cultures
→ Snehal's culture.
→ 2 colonies of ar 3A
~~→ Prep~~

② Prepare ~~Arabinose (& IPTG?)~~ new 20%.

③ Talk to sir about plate readers.

④ Show calculations.

⑤ Check ½ life of GFP + ssRA tag.

⑥ Check promoter induction protocol.

⑦ Dpn1 re-digest" 2 hrs → 2.5 µl w/o Dpn1
→ 2.5 µl w/ Dpn1 → Transform.

⑧ Gel running 0.8% & digest w/ PstI → 0.8% gel.

⑨ Snehal pHyb in Bb ?

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Baranwal

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Induction of pHyb-GFP construct w/ 0.1M IPTG & 0.7% Arabinose

Falcon :-

(1)	C1 Induced PHyb-RBS GFP3A	2ml
(2)	C1 Uninduced —"—	2ml
(3)	C2 Induced PHyb —"—	2ml
(4)	C2 Uninduced —"—	2ml
(5)	C1 Snehal Induced	2ml
(6)	C1 Snehal Uninduced.	2ml

Arabinose stock conc. 20% - Filtersterilized

$C_1V_1 = C_2V_2$

$C_1 = 20\%$

$V_1 = x$

$C_2 = 0.7\%$

$V_2 = (2 + x)$ ml

Store @ 4°C

$20x = 0.7(2+x)$

$20x = 1.4 + 0.7x$

$19.3x = 1.4$

$x = 0.0725$ ml

$x = 72.5$ µl

IPTG stock 1M

$C_1 = 1M$ $C_2 = 1mM$

$1x = 10^{-3}(2 + x)$

$x = 2 \times 10^{-3}$

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Induced @ 5:15 PM
Time pipeted 5:50 pm

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Plate layout : 100 µl

	1	2	3	4	5	6	7	8	9	10	11	12
D	Bl	C1I	C2I	SI	C1U	C2U	SU	C·S				
E	Bl	C1I	C2I	SI	C1U	C2U	SU	C·S				
F	Bl	C1I	C2I	SI	C1U	C2U	SU	C·S				

Bl = SOB

Compcells: C·S

↓

37°C / 180 rpm.

Results:
Excel sheet.

21/10/17 0.8% gel running

Lane 1 : 2 log ladder

2 (Blank)

3 : AraC 3A colony 1 Pst1 dig.

4 : AraC 3A C2 Pst1 dig

5 : LacI 3A C1 Pst1 dig

6 : LacI 3A C2 Pst1 dig

7 : Arac 3A C1 plasmid

8 : Arac 3A C2 plasmid

9 : LacI 3A C1 plasmid

10 : LacI 3A C2 plasmid

11 : pCAT PCR uncut 2µl

12 : RBS PCR uncut 2µl

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Dpn1 digestion of 2µl PCR products LacI & AraC.

Add 5# units of Dpn1 to each tube

↓

Incubate 37°C / 2hrs

↓

Heat inactivate 80°C / 20min.

E & P digestion of 2nd 3A
(Colony 1 & 2 pHyb-RBS-GFPssRA in CAM)

Reaction	Colony 1	Colony 2
DNA	2 µl	2 µl
Buffer 3.1	1 µl	1 µl
EcoR1	0.2 µl	0.2 µl
Pst 1	0.2 µl	0.2 µl
dH_2O	6.6 µl	6.6 µl
	10 µl	10 µl

Colony 1 conc : 62.7 ng/µl
Colony 2 conc : 64.9 ng/µl.

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2X10⁴/1000 µl
20 units in 1 µl

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DpnI rxn. (conc. unknown → PCR prod)

	PCAT-pHyb	RBS pHyb
DNA	3 µl	3 µl
DpnI (~5 units)	0.2 µl	0.2 µl
1X buffer cutsmart	1 µl	1 µl
dH_2O	5.8 µl	5.8 µl
	10 µl	10 µl

Control → ~100ng →

	pCAT	RBS
DNA	2 µl	2 µl
DpnI	0.2 µl	0.2 µl
cutsmart 1X	1 µl	1 µl
dH_2O	6.8 µl	6.8 µl
	10 µl	10 µl

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2A For pHyb in CAM & 3A pHyb, ~~RBS~~ GFPssRA in CAM

2A digestion (HP ~~in CamR~~) & DnaA, FtsZ in Cam

	HP (in μL)	DnaA (in μL) ~~Excess~~	FtsZ (in μ
DNA	4	4	4
EcoRI	0.2	0.2	0.2
PstI	0.2	0.2	0.2
NEB Buffer 3.1	1	1	1
dH_2O	5.4	5.4	5.4
	10μL	10μL	10μL

↓

37°C / 45 mins (water bath)

↓

80°C / 20mins (thermomixer)

Ligation

	HP (in μL)	DnaA (in μL)	FtsZ (in μL)
Insert	1.5	2.1	1.75
BB(PSB1C3)	~~0~~1	1	1
T4 DNA ligase	0.25	0.25	0.25
10X T4 DNA ligase Buffer	0.5	0.5	0.5
dH_2O	1.75	1.15	1.5
	5μL	5μL	5μL

↓

16°C / 6 hours (Thermomixer)

↓

~~Transformation~~

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Master mix for 6 ligations

- 3µL T4 buffer
- 1.5µL T4 ligase
- 7.5µL dH_2O

} 2µL/reaction

3A

1µL part A

1

1

2A

2µL Insert

1µL backbone

2µL Mmix

re-

↓

Transformation → should transform

⇒ 2 replicates of each inoculation

↓

Miniprep

↓

Nanodrop.

	① Thin (ng/µl)	260/280	② fat (ng/µl)	260/280
DnaA Transf	42.5	2.07	109.0	1.99
DnaA 2A	58.8	1.89	98.3	1.94
FtsZ Transf	447.6	1.89	93.1	1.9
FtsZ 2A	55.7	2.02	92.9	1.88
PHyb CAM	104.7	2.03	105.9	1.92
PHyb GFP 3A	105.8	1.99	147.0	1.94
pHyb Lac 3A	45.7	1.93	85.0	1.94
pHyb Ara 3A.	103.0	1.93	104.9	1.91

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Gel running 1% w/ single Pst1 cut

1 → 2 log ladder

3 → DNA A trans T ~ 3.5

DNA A trans F ~ 3.5

DNA A 2A T ~ 3.5

DNA A 2A F ~ 3.5

Fts2 trans T — Diff gel — 3.2

Fts2 trans F — 3.2

Fts2, 2A T — 3.2

Fts2 2A F — 3.2

HP in CAM T — 2.2

HP in CAM F — 2.2

HP GFP T — 3

HP GFP F — 3

HP ~~Ara~~ Ara T — 2 log ladder — 3.2

HP Ara F — 3.2

HP Lac T — Diff gel — 3.~~4~~

HP ~~Lac~~ F — 3.~~4~~

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Double digest E&P

DnaA transformation T

DnaA 2A T

Fts 2 transf T

DFtsZ 2A T

pHyb CAM T

pHyb RBS GFP 3A T

pHyb LacI3A T

★ pHyb AraC 3A T

Reaction

3.1 buffer	1 µl
Dna	4 µl
EcoRI	0.2 µl
PstI	0.2 µl
dH_2O	4.6 µl
	10 µl

↓

2 hrs @ 37°C

20 min / 80°C

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