TABLE OF CONTENS

09/05/17

AIM: Preparation of ~~plates~~ LB Liquid Medium.

MATERIALS REQUIRED:

HIMEDIA LURIA Bertani Broth (M1245-500G)
Erlenmeyer flask
glassware.

PROTOCOL:

1. In 1 L autoclave bottle (orange cap) add 25g LB broth powder, 1000ml ultra pure water. (150 mL 100mL)
LB → (3.75g 2.5g)
2. Swirl to mix. Powder might not mix completely.
3. Replace the cap to the bottle but leave it slightly loose for pressure equalization to occur. Place a fresh piece of autoclave tape on top.
4. Autoclave (121°C, 20 mins) on liquid cycle - be sure to add water to the autoclave basin before starting the cycle! (Creates necessary vapour pressure to prevent the liquid from evapourating in the autoclave)
5. Cool to RT before use. Do not tighten cap until cool.

14.08.16 PLATING CJ Blue transformed cells.

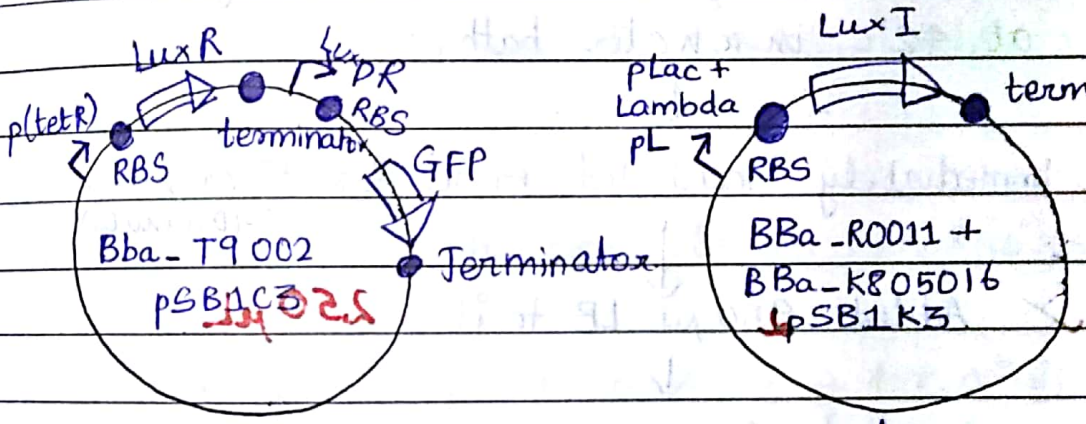
Cells plated : DH5α cells transformed with CJ blue + RBS.

Plate used : Kanamycin plate

Method of plating : pelleting down and plating 50μL.
pelleting down done at 25°c,
3000 rcf, 10 minutes.

ROUGH PROTOCOL FOR T.M EXPTS

EXTRACTION / ISOLATION OF SENDER AND RECEIVER PLASMIDS.



Receiver plasmid Sender plasmid.

AIM: To transform E.Coli DH5α cells with Sender plasmid and receiver plasmid.

MATERIALS REQUIRED : LB, Kanamycin, (LB + Kanamycin) plates, chloramphenicol, (LB + Chloramphenicol) plates, receiver plasmid and competent E.Coli DH5α cells

PROTOCOL: Thawed Sender plasmid DNA on ice

↓

Spun down the plasmids at 9000 rpm for 30 secs in their respective tubes.

↓

Took two 1.5 mL epi and named it with Strain name, name, date, & plasmid name.

(Eg 1. EColi DH5α, Charvee, (date) Sender
2. EColi DH5α, Charvee, (date), receiver)

↓

added 1 µL of Sender plasmid (with Kanamycin antibiotic resistance) into 1.5 mL epi & 1 µL of receiver plasmid (with cam resistance) in another 1.5mL ep

↓

Added 50µL of E.Coli DH5α comp. cells to each of them.

↓

Incubated in ice for 30 mins (meanwhile set water bath to temp of 42°C

↓

Gave a brief heat shock for exactly 60secs at 42°C in a water bath.

↓

Immediately incubated in ice for 5 minutes. (5-10 minutes).

↓

Added 250 µL LB to it 250 µL

↓

Incubated for 2-2½ hrs at 37°C at 200 rpm (take a scotch tape, wrap around the epis and attatch it to the rotor.)

↓

Centrifuged at 25°C, 3000 rcf, 10 mins (keeping equal amount of water on another ep on the opposite side for balancing).

Not read here because we can place two epis opp. to each other

↓

Set pipette to 950µL and remove the Supernatant out of each of the epi.

→ Discard 950µL supernatant

↓

Resuspend the pellet in 50µL LB using a 200µL pipette set to 50 µL.

transformants (Sender)
plated 50 µL of the transformants without pelleting down (for Receiver plasmid) and stored rest in 4°C

plate this 50 µL volume "transformed" cells of E.coli DH5α on LB/Kanamycin plate.

plate the cells transformed with Sender plasmid on LA + Kanamycin plate. (50 µL of cells) and Label them properly (pelleted)
(&)
plate the cells transformed with receiver plasmid on LA + Chloramphenicol plate (no pelleted) and label it properly (Channel, date, LA/cam, Receiver).

MINIPREP PLASMID EXTRACTION [Sender and Receiver plasmid]

Step 1 : Inoculating transformed colony for miniprep.

Took 2 mL LB - Cam (25 µg/mL) and
falcon 1 ↙ 2 mL LB - Kan (25 µg/mL) in 2 Separate falcon
falcon 2 ↙

↓

picked Sender plasmid - transformed colony into falcon 2 with 10µL tip.

picked Receiver plasmid - transformed colony into falcon 1 with 10µL tip

↓

Incubated at 37°C, 200 rpm for 18 hrs.

Step 2: Miniprep plasmid extraction: (Sender + Receiver plasmid)

falcon 1 (Receiver plasmid transformed cells, 2mL)

falcon 2 (Sender plasmid transformed cells, 2mL)

↓

pipetted 1 mL culture into 2 epi of 1.5 mL each and Labelled

pipetted 1 mL culture in 2 epis of 1.5 mL each and Labelled.

↓

centrifuged @ 8000 rpm (6800 rcf) for 3 mins @ 25°C

→ Discard Supernatant

Resuspended pellet in 125 µL P1 buffer (to which RNAase and Lyse blue is added and kept in 4°C)

make sure lyseblue is dissolved by shaking vigourously

↓

Pooled them together into a 1.5 mL eppi

↓

Added 250 µL P2 buffer and mixed well by Inverting the tubes (3 mins) (DO NOT MIX VIGOROUSLY)

↓

Added 350 µL N3 buffer and mixed by inverting the tubes 4-6 times

- WEAR GLOVES WHILE HANDLING BUFFERS
- Check P2 and N3 for salt ppt. Redissolve any ppt by Warming to 37°C

→ Obtained by the solution when P2 buffer was added.

mixed until all trace of blue has gone

↓

Centrifuged for 10 mins @ 17900 rcf / 13000 rpm @ 25°C

↓

↓ → Discard pellet

Applied 800 µL of supernatant from prev. step to QIAprep 20 Spin column by pipetting

↓

centrifuged at 17900 rcf for 60 sec

↓ → Discarded flow through

Added 0.5 mL PB buffer to the column

↓

centrifuged at 17900 rcf for 60 sec

↓ → Discarded flow-through

↓

Added 0.75 mL PE buffer to the column

↓

Centrifuged at 17900 rcf for 60 sec

↓ → Discarded flow-through

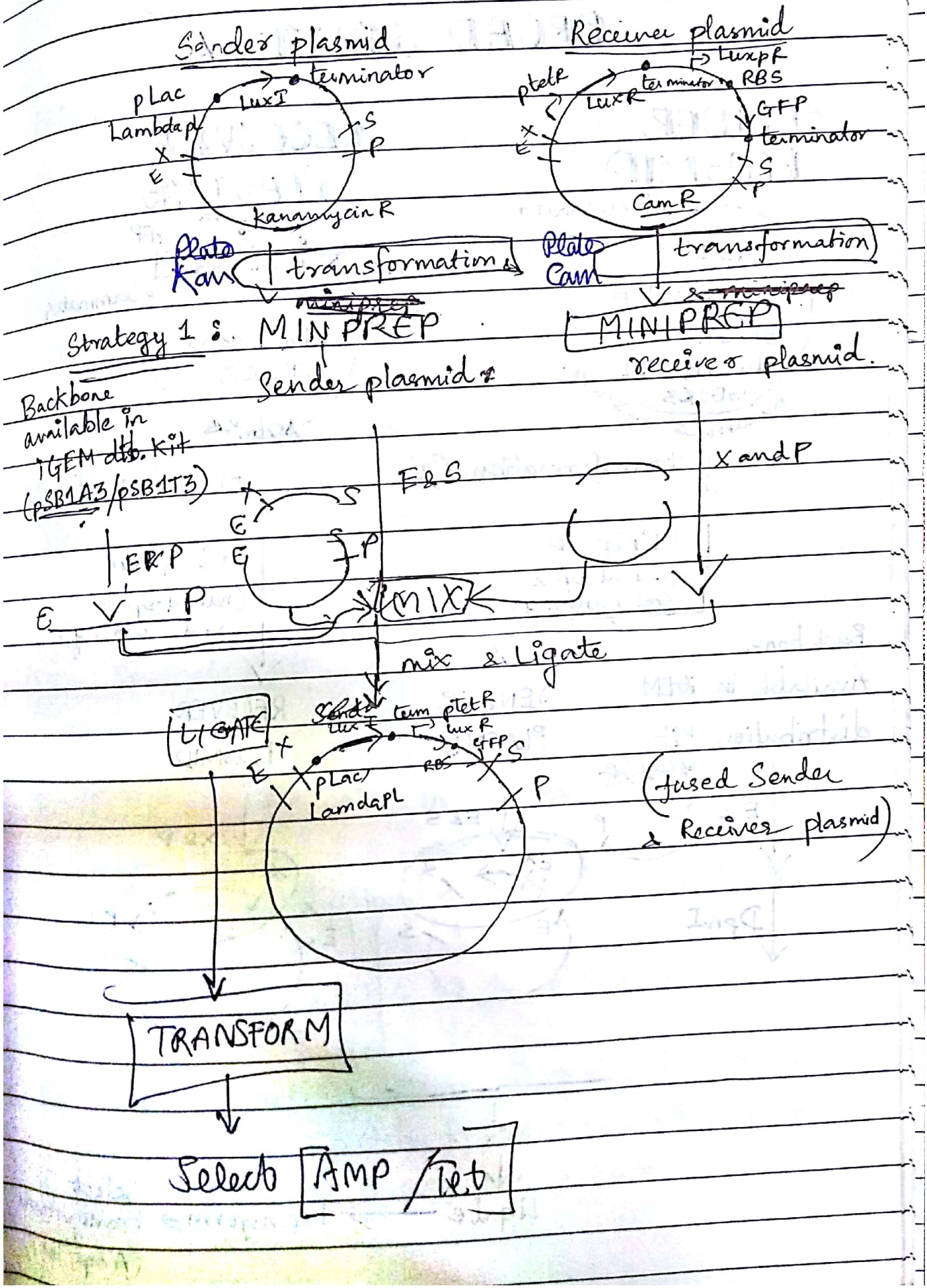
↓

Centrifuged @ 17900 rcf for 60 sec to remove residual wash buffer.

↓

transfered the column into 1.5 mL eppi

BROAD STRATEGY

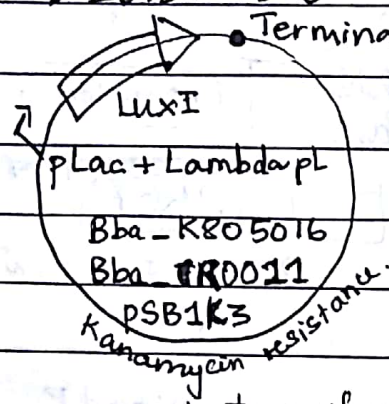


21 Aug 17

TERMINATION MODULE PROTOCOLS

BROAD STRATEGY

SENDER PLASMID



transformation (I)

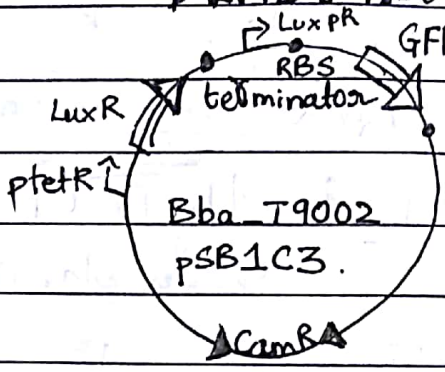
Miniprep
(nanodrop & Gel running)

Backbone Available in iGEM distribution kit

E & P

DpnI

RECEIVER PLASMID



transformation

miniprep
(nanodrop & Gel running)

SENDER PLASMID

E & S

RECEIVER PLASMID

X & P

Mix

Ligate → Transform → Select transformants (Amp/Tet)

23 Aug 17

TRANSFORMATION OF SENDER & RECEIVER PLASMID INTO DH5α CELLS.

PROTOCOL: Thawed sender plasmid, receiver plasmid and competent cells on ice.

↓

Spun down plasmids @ 9000 rpm 30secs

↓

Labelled two epis.

I. Sender, 23 Aug 17, Charvee, DH5α

II. Receiver, 23 Aug 17, Charvee, DH5α.

↓

(I.) Add 1 µL Sender plasmid (II.) 1 µL receiver plasmid

Freshly made. → 50 µL comp. cells 50 µL Comp Cells.

↓

Incubate epis (I & II) in ice for 30 mins

↓

brief heat shock (60secs) @ 40°C in Water bath

↓

Immediately incubated in ice for (5–10 mins).

↓

Added 250 µL LB to epis (I & II)

↓

Incubated for 2hrs @ 37°C @ 200 rpm (Took Scotch tape, wrapped around the epi & attatched to rotor

↓

6/10/17 Gel running of miniprep product

6/10/17

① Digestion of Sender & Receiver

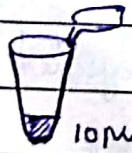
Reaction : Mastermix

5 µL	NEB Buffer 3.1	(for 25 µL)
0.5 µL	EcoRI	
19.5 µL	dH_2O	

6 reactions → 30 µL
(2×Ch, 3×Jy, 1×Za)

Calculations :

23.4 µL dH_2O	30 mL
0.6 µL EcoRI	Enzyme Master
6 mL NEB Buff 3.1	Mix

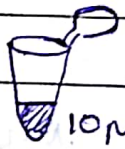


10 µL

Sender plasmid (5 µL)

+

5 µL (Enzyme Master mix)



10 µL

Receiver plasmid (5 µL)

+

Enzyme master mix (5 µL)

↓ digestion

for 30 mins, 37°C

↓

Heat inactivation for 20 mins, 80°C

↓

Short Spin

↓

Vortex

↓

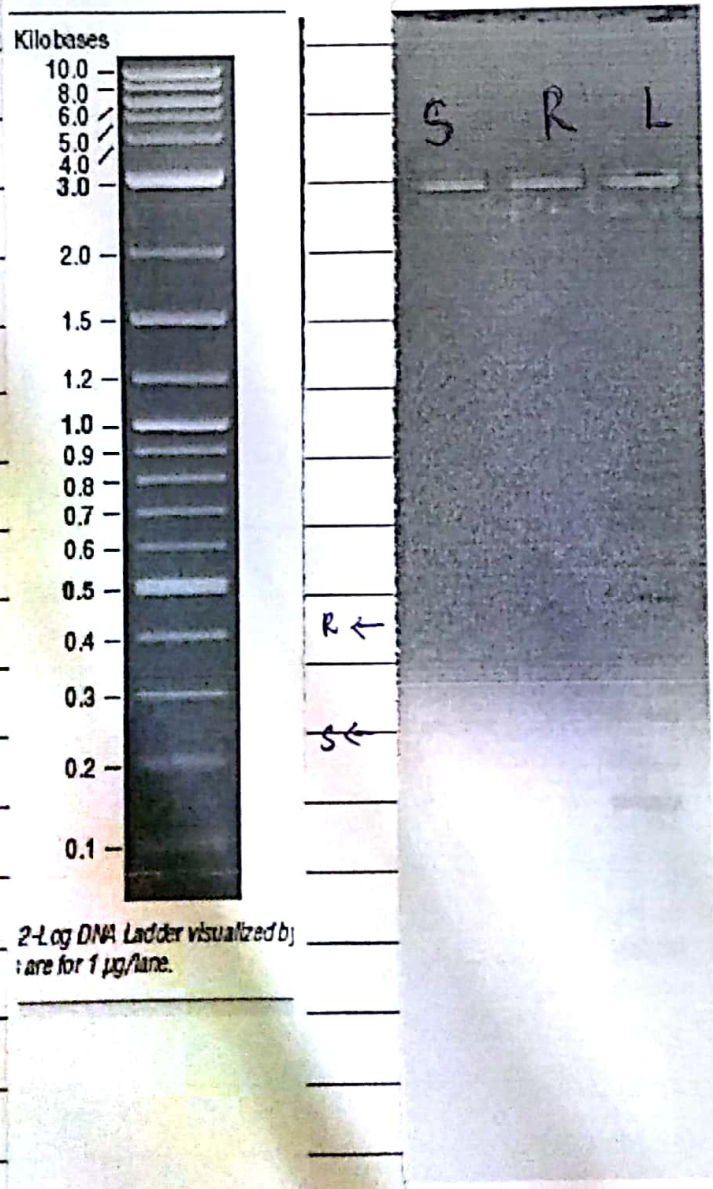
Short Spin. → Ready to be loaded

* Gel: Agarose Gel (1%) + EtBr (2 drops).
* Load Sample (5µL) + 6x loading dye (1µL) in each well.

Ladder	4V2	4V1	J1	J3	J2	Zak	← Sample Order
2 log DNA 2µL	(S) (5µL + 1µL 6x LD)	(R)	(pCAT)	(ACP 14-9)	(ACP 5-10)		

* Ran the gel for one hr @ 80V

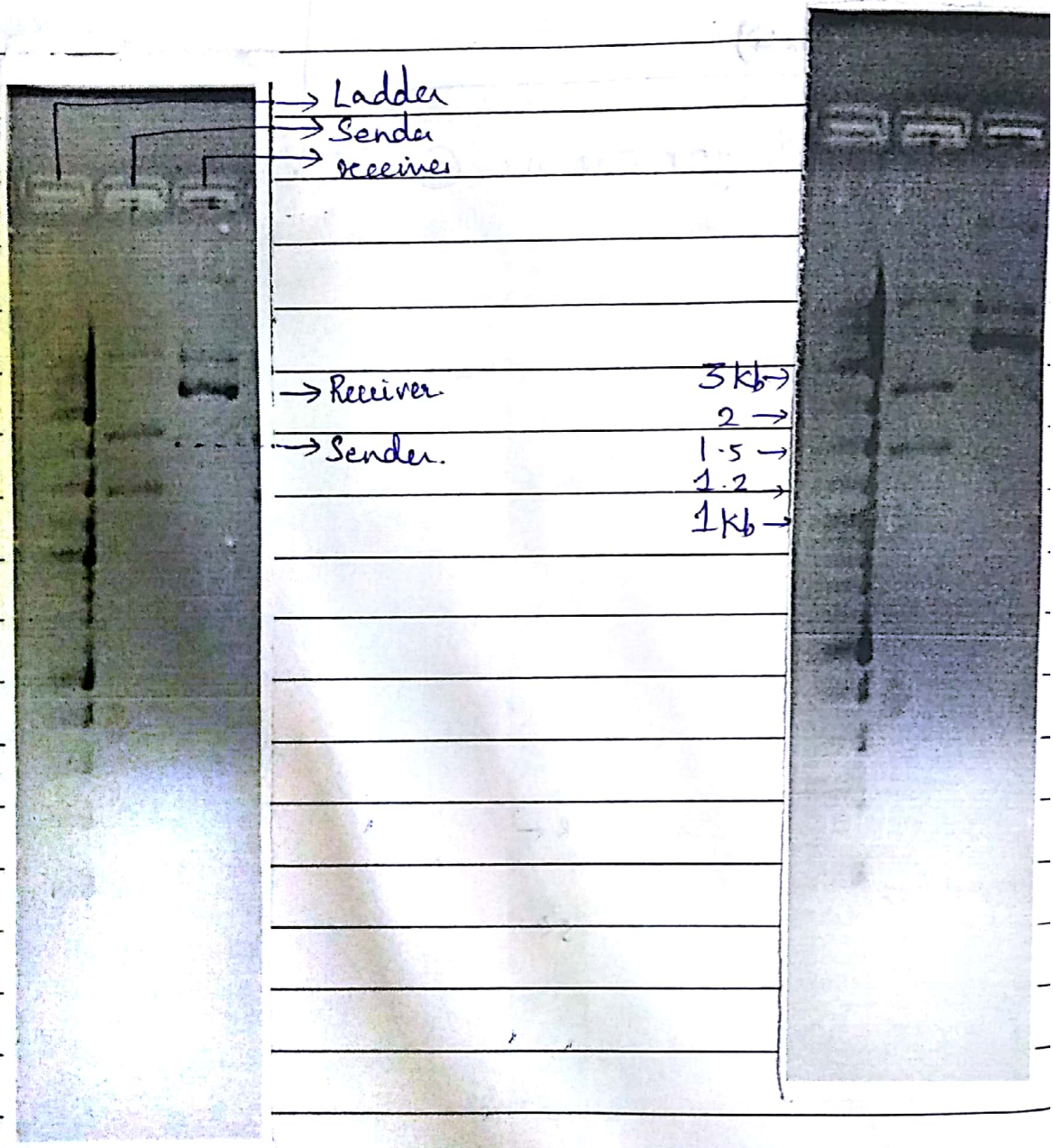
Result:
of 1st Gel Running.



2-Log DNA Ladder visualized by
are for 1 µg/lane.

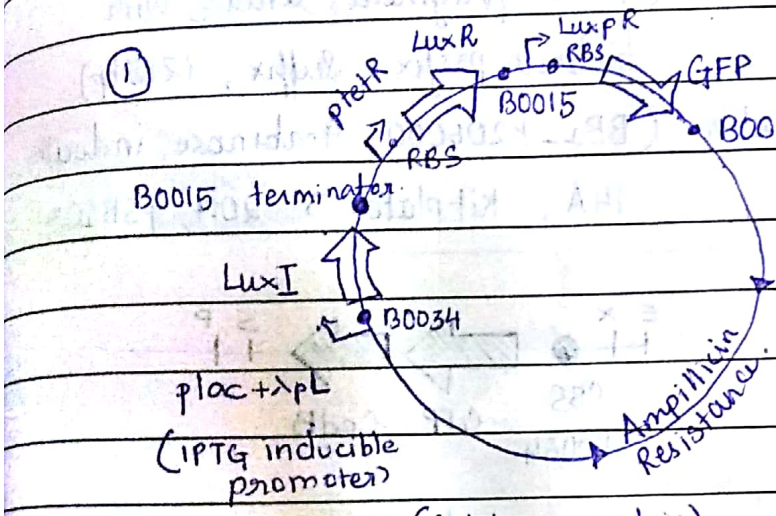
Expect length of R : 4015 bp.
Expected length of S ≈ 2000 bp.

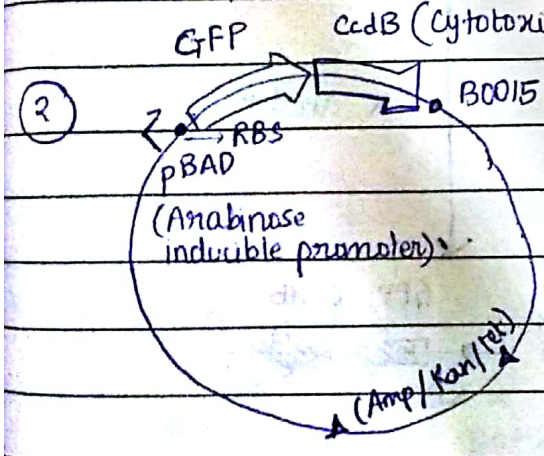
RESULT OF GEL RUNNING DONE ON 6/10/17.

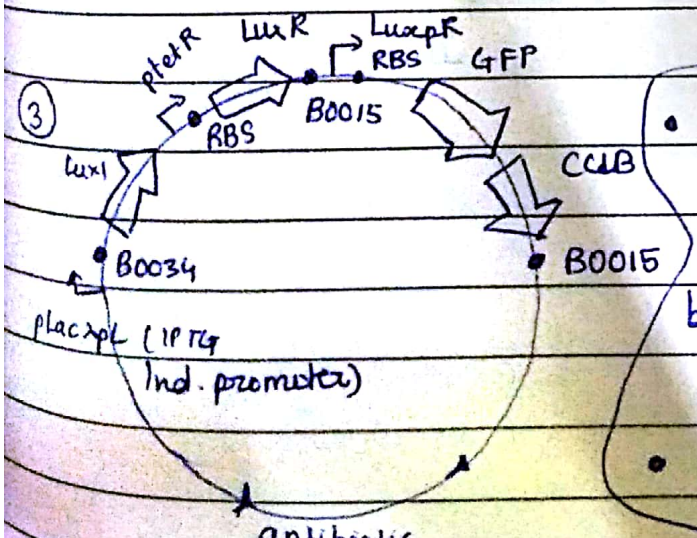


3A ~~Assembly~~

Constructs I want to prepare.







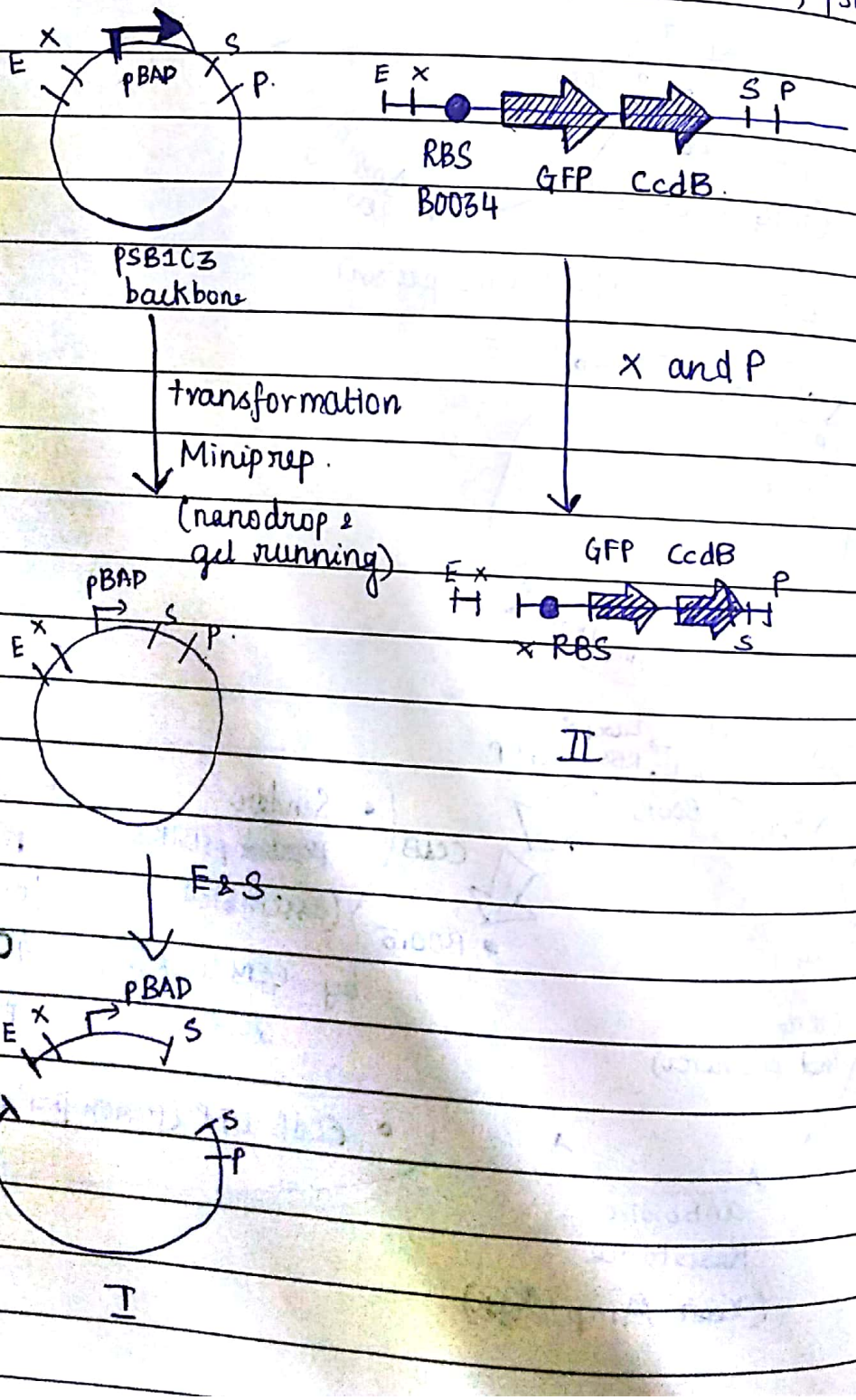
- Sender ~~pSB1A3~~ pSB1K3 (assembled by iGEM IISER Pune 2015)
- Receiver ~~pSB1C3~~ (BBa_F2620 40 3 plate - 2017) pSB1C3
- ECdB-GFP (fusion protein)

To prepare construct ①, for broad protocol, refer Pg 46.

To prepare construct ②,

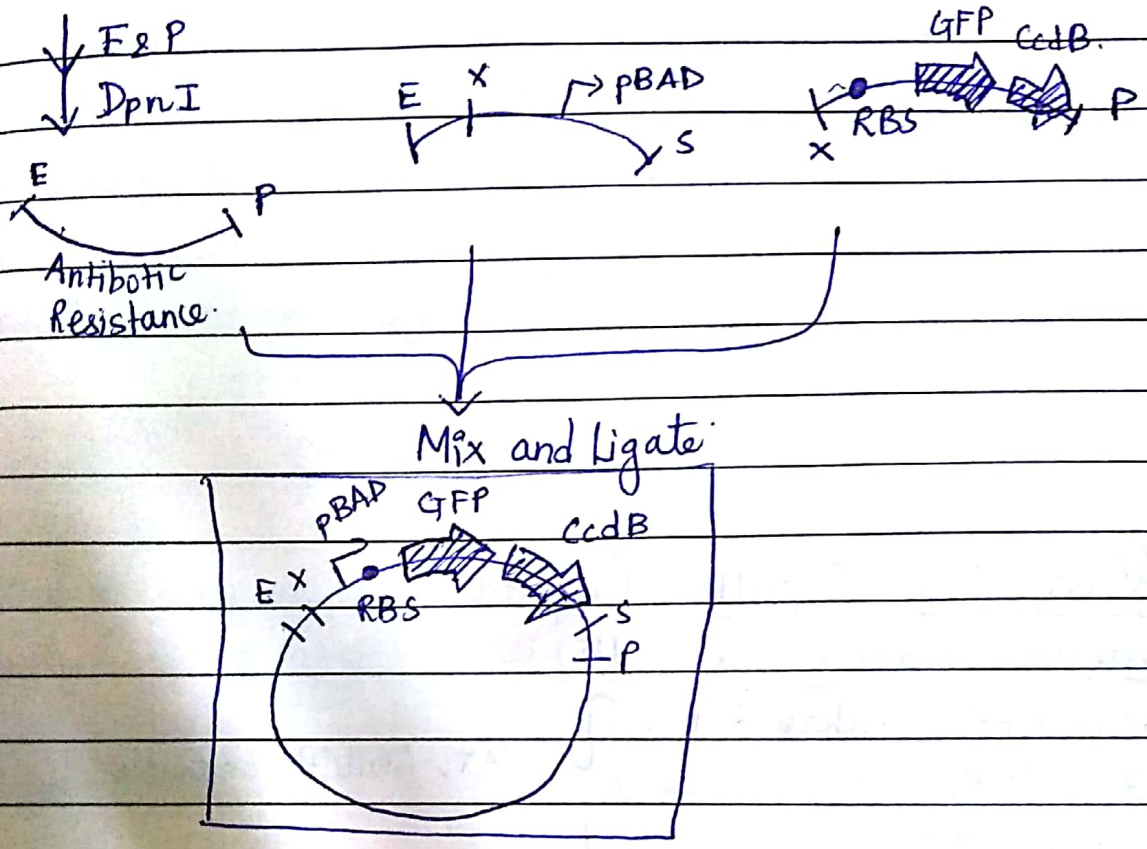
Required parts : RBS-GFP-ccdB (Gblock fragment, linear, with biobrick prefix & suffix, 1213 bp)

pBAD promoter (BBa_K206000, Arabinose inducible 14A, Kit plate: 3 2017, pSB1C3)



plasmid backbone.

pSB1A3/pSB1A3/pSB1K3



*To prepare construct (3):

~~Solution~~ Required parts:

3A assembly protocol

Part A	Part B	backbone
Sender	receiver	Ampicillin pSB1A3
46.4 ng/μL	71.8 ng/μL	25 ng/μL
46.4×10^{-9} g	71.8×10^{-9} g	25×10^{-9} g
~~0.9933~~ $\times 10^{-18}$	~~2.4~~ $\times 10^{-18}$ g	2.325×10^{-18} g
3.237	4.33	
14.33×10^{9} moles/μL	16.58×10^{9} moles/μL	10.75×10^{9} moles/μL

3 μL	2.6 μL	4 μL
+	+	+
1 μL dH_2O	1.4 μL dH_2O	0 μL dH_2O

Mastermix, Required DNA volume = 10 μL.

7.5 μL	6.5 μL	10 μL backbone
+	+	+
2.5 μL dH_2O	3.5 μL dH_2O	0 μL dH_2O

Mastermixes

EcoRI - 0.2 μL	XbaI - 0.2 μL	E.coRI - 0.2 μL
SpeI - 0.2 μL	PstI - 0.2 μL	PstI - 0.2 μL
NEB 2 - 2 μL	NEB 3.1 - 2 μL	DpnI - 0.2 μL
BSA - 2 μL	dH_2O - 7.6 μL	NEB 3.1 - 2 μL
dH_2O - 5.6 μL	10 μL	dH_2O - 7.4 μL
10 μL		10 μL

Calculation

receiver 1945 bp

650 x 1945 (da/basepair) (bp)

1264250 da

$\times 1.66 \times 10^{-24}$ g/da

2098655×10^{-24} g

2.1×10^{-18} g

Sender

55bp + 798 bp + 12 bp = ~~865~~ bp

650 da/bp x 865 bp = 562250

$562250 \times 1.66 \times 10^{-24}$ g

$= 9.33335 \times 10^{-24}$ g

$= 0.9333 \times 10^{-18}$ g

Receiver

4015 x 650 $\times 1.66 \times 10^{-24}$

4.33×10^{-18}

Sender

~~0.~~ 3.237×10^{-18}

PSB1A3

2155 x 650 x 1.66 $\times 10^{-24}$

$= 2.325 \times 10^{-18}$

10µL DNA + 10µL M·Mix (part A)

10µL DNA + 10µL M·Mix (part B)

10µL DNA + 10µL M·Mix (backbone)

↓

digestion 37°C for ~~30 min~~ 3 hrs

↓

Heat inactivation for 20mins @ 80°C.

↓

Stored @ -20°C.

Running gel to check digestion efficiency

	17.4 ng/µL	R 23.335 ng/mL	PB 12.5 ng/µL
final conc.	17.4 ng/µL	23.335 ng/mL	12.5 ng/µL
Volume : (for ≥ 100ng)	6µL S (digest) 1µL LodB	5µL R (digest) +1µL LB	~~6µL~~

Ladder	Sender digest	Receiver Sender Uncut	Receiver digest	Receiver Uncut
2µL	6µL	3 µL	5µL	2µL
	1µL B	1µL LB	1µL LB	1µL LB.

Sender

2204 bp (backbone)

800 bp (Insert).

Receiver

2070 bp (backbone)

1945 bp. (Insert)

Ligation :

	Sender	Receiver	PB
	1 µL	1·3 µL	0.7 µL

Ligation :

Mastermix

23/10 Inoculated for Miniprep Sender & Receiver

Falcon 1 : One colony Sender + 2mL SOB + 2µL Kanam (35

Falcon 2 = One colony receiver + 2mL SOB + 2µL Cam(

- Kept Overnight @ 37°C for 12-16 hrs

23/10/17

2A assembly

(To subclone Sender pLac/Lamda pL ~~into~~ -LuxI into pSB1C3)

↓ BB. ↓ Insert

Digestion

① Insert : Sender : pLac/LamdapL-LuxI (15 μM : Refer Pg:)

→ 5 μL.

② Enzyme master mix (E&P) → 5 μL taken

→ MilliQ:

→ EcoRI :

→ PstI :

→ NEB :

} Prepared.

↓

10 μL (5 μL Sender + 5 μL enzyme mastermix).

↓

Kept / incubated @ 37°C for 1 hr 15 mins

↓

Heat inactivation for 20 mins @ 80°C

↓

Stored @ -20°C

Final Conc. of digested Sender : $\frac{15\ \mu M}{2} = 7.5\ \mu M$.

24/10/17.

Backbone E & P digestion.

E.P

1 µL of digested pSB1C3 was already prepared upto final conc of 5 µM.

Ligation of Sender (Insert) into pSB1C3 (backbone)

Calculation:

→ 2A assembly ligation requires Insert : Backbone :: 3:1 (molarity)

∴ 5µ moles of Backbone requires 15µmoles of Insert (1 ML of 5µM BB)

⇒ Insert volume required = 7.5µM x 2µL = 15 µmoles

Ligation Mix

- T4 ligase = 0.5 µL
- T4 Ligase = 1.0 µL Buffer
- MilliQ = 2.5 µL

Total Vol: 4µL

2µL → 2µL Insert (Sender) + 1µL pSB1C3 (BB) + 2µL Ligation mix (prepared 4µL)

2µL

Jyothish's Rxn

2µL G13
1µL ts-purple
2µL Ligation mix

↓

Incubated overnight @ 16C for 12hrs.

24/10/17

Transformation of Sender-Receiver 3A product to (cloned Sender & Receiver into pSB1A3 backbone)

The 3A product from Sender-Receiver-pSB1A3 ligation was transformed into comp cells of MG1655 strain (use SOC for growth) (For Transformat^n protocol, look Pg: 47)

↓

Incubate the Transformed cells @ 37°C for 5hrs

↓

plated the transformed cells in Amp (35µg/ml) LA plates.

↓

kept/incubated overnight @ 37°c.

↓

lawns observed in both the plates (-ve control & 3A:assembly product transformed cells).

25/10/17 Did Transformation of

① 3A assembly ligated product (Sender-Receiver-pSB1A3)

② 2A assembly product (Sender-pSB1C3)

into MG1655 comp cells and plated in

① Amp/LA

② Cam/LA

↓

observed colonies on 26/10/17

↓

26/10/17 Inoculation For miniprep.

~~Minipreppe~~

Inoculated 1 colony of 2A assembly product into 2mL SOB, 2µL Cam. in 50mL falcon (S1)

Inoculate 2 colonies of 3A assembly product in two diff 50 mL falcons (with 2mL SOB, 2µL Amp each) (SR1, SR2)

↓

Inenbated for 6 hrs @ 37°C

↓

Miniprepped (For miniprep protol Refer:)

↓

~~S1~~	conc.	$A_{260/280}$	$A_{260/230}$
S1	95.6	1.97	2.28
SR1	46.6	2.01	2.17
SR2	46.1	2.06	2.06

Meanwhile, a miniprep of Sender (pSB1K3) & Receiver (pSB1C3) was also done

Sender	155	1.87	2.06
Receiver	99.9	1.96	2.37

2A assembly G-block gene fragment (GFP-ccdB fusion protein) into pSB1C3 plasmid backbone.

Calculation:

1:3 :: Backbone : Insert.

5 μ-moles of Backbone need 15 μ moles of Insert

(1 μl pSB1C3 backbone)

5 moles/μl

Insert : ccdB - GFP fusion protein : 1214 bp = 789100 da

: 10 ng/μl : $10 \times 6.022 \times 10^{14}$ da/μl

$= \frac{6.022 \times 10^{15}}{789100}$ moles/μl

$= 7.6314 \times 10^{9}$ moles/μl

$\frac{\pi x^2}{10} =$

$7.7 \times \frac{x}{10} = 5 \Rightarrow x = \frac{150}{7.7} \approx 20$

$x = \frac{50}{7.5} = 6.6$

$T_4 = 0.25$

T_4 ligase Buffer : 0.5

dH_2O = N/A (1.25)

Insert : 2 μl + 1.25 μl = 3.25 μl

3.25 μl of insert = 15 μ Moles

3.25 X (dig) digested = 15

insert conc

digested insert conc $= \frac{15}{3.25} = 4.6\ \mu M$

Insert initial conc = $7.7\ \mu M$

Digestion mastermix : 0.2 µL EcoRI
0.2 µL Pst1
1 µL NEB 3.1

$\frac{7.7 \times x}{10} = 4.6$

$x = \frac{4.6 \times 10}{7.7} = \frac{46}{7.7} \approx 6\ \mu L$

$\therefore dH_2O = 10\ \mu L - (0.2 + 0.2 + 1 + 6)\ \mu L$
$= 10\ \mu L - 7.4\ \mu L$
$= 2.6\ \mu L$.

Ligation : (CcdB i

T4 ligase = 0.5 ÷ 0.25 µL (directly in Small epi)
T4 ligase buffer = 0.5 µL
Insert (digested) = 3.25 µL
pSB1C3 (digested) = 1 µL

↓

Ligated overnigh @ 16°C

↓

Transformed into MG1655 comp cells

↓

Incubated cells @ 37°C
with 250 µL SOB for 5 hrs

26-10-17 : Get Running to verify the 2A and 3A assembly products, miniprepped Sender & Receiver.

Digestion (Single Cut)

5 µL (Sample) + 5 µL (Mastermix)

→ S1, SR1, SR2, Sender, Receiver have to be digested

∴ Require 5x Mastermix of total volume 25 µL (Mastermix for 5 Reactions).

= 5 µL NEB Buffer 3.1
0.5 µL EcoRI
19.5 µL dH_2O.

↓

for each reaction, used 5 µL MM & 5 µL of Sample

↓

Incubated the epi @37°C for 45 mins.-1hr

↓

Stored digested product @ −20°C

1. 2log DNA ladder
2. G13-tsp Tet ②
3. G13-tsp ① Tet
4. pL plaft Cam-EcoRI
5. pL plaft Acp
6. pCAT Acp
7. pCAT tet
8. plaft
9. 2 log DNA ladder
10. Sender (Kana) Uncut
11. Sender Kana cut
12. Sender-Receiver (Uncut)
13. Sender-receiver cut
14. S1 uncut (Sender in pSB1C3)
15. S1 cut

Sender (pSB1K3) : 850bp + 2204 bp = 3054

Sender (pSB1C3) : 55+ 798 + 2070 bp = 2920

Receiver (pSB1A3) : (850+ 1945)bp + 2155bp

-Sender : = **4950**