protocol template(partly copied from Alex's labnote)

Basic information about plasmid

plasmidA
pSB1A3-mRFP:→backbone
gB_iRFP:→iRFP
oligo_A
sfGFP

plasmidB
pSB4K5-mRFP:→backbone
gB_lp-ecfp-sopB_2
gB_lp-ecfp-sopB_1
oligo_B_1
oligo_B_2
cI-1
DT2
sopC

plasmidC
pSB3C5-mRFP:→backbone
oligo_ParS
DT1
TetR1
oligoC
gB_lp-ecfp-parB_1
gB_lp-ecfp-parB_2

plasmidD
~~pSB4K5-mRFP:→backbone~~
pSB3C5-mRFP:→backbone
oligo_ParS
DT1
TetR2
oligo_D
cI-2
DT3
gB_lp-ecfp-parB_1
gB_lp-ecfp-parB_2

Plasmid	template	PCR type	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
A	sfGFP	BioBrick PCR	f017	53	f018	51.8	52	789
C	DT_1	BioBrick PCR	f026	54.9	f024	55.1	55	189
D	DT_1	BioBrick PCR	f026	54.9	f024	55.1	55	189

B	DT_2	BioBrick PCR	f024	55.1	f025	57.1	56	189
Df	DT_3	BioBrick PCR	f024	55.1	f046	62.1	59	189
A	pSB1A3	BioBrick PCR	f004	59.9	f003	60.1	60	2155
C	pSB3C5	BioBrick PCR	f004	59.9	f003	60.1	60	2738
D	pSB3C5	BioBrick PCR	f004	59.9	f003	60.1	60	2738
B	pSB4K5	BioBrick PCR	f004	59.9	f003	60.1	60	3419
C	TetR	BioBrick PCR	f027	70.8	f045	68.5	68	660
B	cI_2	BioBrick PCR	f057	66.6	f023	70.1	68	750
D	cI_1	BioBrick PCR	f044→f107	66.6	f023	70.1	68	780
D	TetR	BioBrick PCR	f027	70.8	f082	68.5	69	690
B	CFPsopB_1	gBlock PCR	f020	55.9	f021	53.3	55	977
B	CFPsopB_2	gBlock PCR	f001	60.1	f019	62	60	851
C	CFPparB_1	gBlock PCR	f029	58	f030	60.5	60	903
D	CFPparB_1	gBlock PCR	f029	58	f030	60.5	60	903
C	CFPparB_2	gBlock PCR	f031	60.5	f002	60.6	60	921
D	CFPparB_2	gBlock PCR	f031	60.5	f002	60.6	60	921
A	iRFP	gBlock PCR	f001	60.1	f016	56.9	60	1009
B	oligo_B_1	oligo no-template PCR	f058	62.8	f059	62.8	63	116
C	oligo_C	oligo no-template PCR	f062	65	f063	65	65	129
B	oligo_B_2	oligo no-template PCR	f060	65.9	f061	65.9	66	116
D	oligo_D	oligo no-template PCR	f055	69.3	f056	69.3	69	114
A	oligo_A	oligo no-template PCR	f042	71	f043	71	71	137
C	oligo_parS	oligo no-template PCR	f032	77.9	f033	77.9	78	112
D	oligo_pa	oligo	f032	77.9	f033	77.9	78	112

	rS	no-template PCR						
B	sopC	others	f049	55	f050	55.5	55	580

	plasmid A	**plasmid B**	**plasmid C**	**plasmid D**
pSB1A3-mRFP	backbone			
pSB4K5-mRFP		backbone		backbone
pSB3C5-mRFP			backbone	
pSB1C3_sfGFP	insert			
pSB1A3-cI		insert		insert
pSB1C3_doubleterminater		insert	insert	insert
pSB1A3-TetR			insert	insert
E.coli K-12F (pEE29=pmk755)		insert		
gB_iRFP	insert			
gB_lp-ecfp-parB_1			insert	insert
gB_lp-ecfp-parB_2			insert	insert
gB_lp-ecfp-sopB_1		insert		
gB_lp-ecfp-sopB_2		insert		
oligo_A	insert			
oligo_B_1		insert		
oligo_B_2		insert		
oligo_ParS			insert	insert

oligo_C			insert	
oligo_D				insert

shinchoku list (parts)

	restriction enzyme reaction	**PCR**	**electrophpresis**	**column purification**
pSB1A3-mRFP	9/13 tajima	9/13 tajima	9/14 tajima	9/14 tajima
pSB4K5-mRFP	9/13 tajima	9/13 tajima	9/14 tajima	9/14 tajima
pSB3C5-mRFP	9/13 tajima	9/13 tajima	9/14 tajima	9/14 tajima

	restriction enzyme reaction	**PCR**	**electrophoresis**	**column purification**
pSB1C3 sfGFP	9/12 goya	9/12 goya	9/13 koga	9/14 tajima
cI	9/13 tajima	9/13 tajima	9/13 tajima	9/14 tajima
pSB1C3 DT	9/12 goya	9/12 goya	9/13 koga	9/14 tajima
TetR	9/12 goya	9/12 goya	9/13 koga	9/14 tajima
E.coli K-12 F plasmid (JM109) ??	9/14 tajima	9/14 tajima	9/14 tajima	

	gblock PCR	**electrophoresis**	**column purification**
gB_iRFP	9/11 goya	9/11 goya	9/11 goya
gB_lp-ecfp-parB_1	9/11 goya	9/11 goya	9/11 goya
gB_lp-ecfp-parB_2	9/11 goya	9/11 goya	9/11 goya
gB_lp-ecfp-sopB_1	9/11 goya	9/11 goya	9/11 goya
gB_lp-ecfp-sopB_2	9/11 goya	9/11 goya	9/11 goya

	oligo no-template PCR	electrophpresis using acrylamide gel	gel purification	column purification
oligo_A	9/9 nishizawa/tajima	9/10 goya		9/11 goya
oligo_B_1	9/9 nishizawa/tajima	9/10 goya		9/11 goya
oligo_B_2	9/9 nishizawa/tajima	9/10 goya		9/11 goya
oligo_ParS	9/9 nishizawa/tajima	9/10 goya		9/11 goya
oligo_C	9/9 nishizawa/tajima	9/10 goya		9/11 goya
oligo_D	9/9 nishizawa/tajima	9/10 goya →looks short →Should we try different oligo sequences?	9/11 goya	

Outline of construction

the plasmid consists of (1)backbone (2)gblock-derived insert (3)oligo-derived insert (4)plasmid-derived insert

Bacterial transformation protocol

- Add 1uL of plasmid DNA or distilled water (NC) into 25uL of DH5a competent cell.
- Incubate on ice for 30 min.
- Heat-shock for 45 sec at 42℃ on the heat block.
- Add 250uL of SOC medium to each sample.
- incubate at 37℃ for 1h.
- Centrifuge at 10,000rpm for 1 min to pellet cells.
- Discard supernatant to 100uL and resuspend pellets.
- Spread on LB plates that contain appropriate antibiotics.
- Incubate at 37℃ for 16h.

OE-PCR

template mixture list↓

[Plasmid A]

number	sample	concentration(ng/uL)	amplicon size
A1	iRFP	44.2	1009
A2	oligo_A*	44.2	137
A3	sfGFP	99.4	789

[Plasmid B]

number	sample	concentration(ng/uL)	amplicon size
B1	gB_lp-ecfp-sopB_2	20.5	851
B2	gB_lp-ecfp-sopB_1	47.1	977
B3	oligo_B_1*	35.0	116
B4	oligo_B_2*	63.0	116
B5	cl_1	227.3	750
B6	DT_2*	49.5	159
B7	sopC	29.4	571

[Plasmid C]

number	sample	concentration(ng/uL)	amplicon size
E1	oligo_parS*	49.1	112
E2	DT_1*	35.3	189
C1	TetR1	66.4	660
C2	oligo_C*	29.2	129
E3	gB_lp-ecfp-parB_1	18.0	903
E4	gB_lp-ecfp-parB_2	7.6	921

[Plasmid D]

number	sample	concentration(ng/uL)	amplicon size
E1	oligo_parS*	49.1	112
E2	DT_1*	35.3	189
D1	TetR_082(TetR2)	224.8	690
D2	oligo_D*		114
D3	cl_2	208.9	780
D4	DT_3*	54.0	189
E3	gB_lp-ecfp-parB_1	18.0	903
E4	gB_lp-ecfp-parB_2	7.6	921

OE-PCR program: **2kb ≦ expected amplicon size**

steps	℃	time	
predenature	98	1min	
denature	98	10sec	↑
annealing	60	15sec	**30** cycle
extension	68	5min	↓
hold	16	∞	

OE-PCR program: **expected amplicon size ≦ 2kb**

steps	℃	time	
predenature	98	1min	
denature	98	10sec	↑
annealing	60	15sec	**30** cycle
extension	68	3min	↓
hold	16	∞	

reaction composition:

components	conc.	volume (uL)	final conc.
distilled water		6.2	
5xPrimeSTAR GXL Buffer	5x	2	1x
dNTPs	2.5mM	0.8	200uM each
F primer	10uM	0.2	0.2uM

R primer	10uM	0.2	0.2uM
Template Mixture	*	0.4	
PrimeSTAR GXL DNA polymerase		0.2	
total		**10**	

oligo no-template PCR

to obtain these cloning part below by oligo no-template PCR

PCR program:

steps	℃	time	
predenature	94	2min	
denature	98	10sec	↑
extension	68	10sec	↓ 12 cycle
hold	16	∞	

reaction composition:

components	conc.	volume (uL)	final conc.
distilled water		11.6	
5x PCR Buffer		4.0	1x
dNTPs	2.5mM	1.6	0.5mM each
F primer	10uM	1.2	0.6uM
R primer	10uM	1.2	0.6uM
Prime STAR GXL		0.4	
total		20	

restriction enzyme reaction

plasmid	XuL (-200ng)
bufferH	2ul
restriction enzyme1	0.5ul
restriction enzyme2	0.5ul
SAP	1uL

BSA(0.1%)	2ul
TritonX(0.1%)	2ul
DW	12-X ul
total	20ul

incubated at 37℃ for at least 1hr, then at 65℃ for 5min to heat-inactivate.
After the digestion, the sample was directly used as a PCR template.

PCR(template: plasmid)

PCR program:

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	5min	↓
hold	16	∞	

reference:
【if amplicon ≦10 kb】
3step
[98℃ 10 sec.
55℃ or 60℃(*1) 15 sec.
68℃ 1 min. / kb]
×30cycles

2step
[98℃ 10 sec.
68℃ 1 min./kb]
×30cycles

(*1)
if Tm >＝55℃→annealing temperature should be 60℃
if Tm＜55℃→annealing temperature should be 55℃
◆
・first you should try 3 step PCR.
If you see smear bands or strange bands, you should increase annealing temperature or try 2 step PCR.
・if the amplicon is GC rich, you should try 2step PCR

reaction composition:

components	conc.	volume (uL)	final conc.
distilled water		16	

10x PCR Buffer for	10x	2.5	1x
dNTPs	2mM	2.5	0.2mM each
MgSO4	25mM	1.5	1.5mM
F primer	10uM	0.5	0.2uM
R primer	10uM	0.5	0.2uM
plasmid	1ng/uL	1	1ng/25uL
Enzyme		0.5	
total		25	

After the reaction, treat with 0.5uL of DpnI at 37℃ **o/n**

gBLock PCR

PCR program:

template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	11.2ul
Total	20uL

2.centrifuge
3.PCR

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	12 cycle
extension	68	1min	↓
hold	22	∞	

colony PCR

x2 emerald amp	5ul	Pipette 10uL each
DW	4.6ul	
primer(10uM each)	0.2ul each	

Electrophoresis

for normal electrophoresis
1% agarose-TAE gel / 100V 20min

for gel purification
1% LMP agarose gel / **50V 40 min**

for acrylamide gel
8% acrylamide gel / 100V 60min

column purification :

1. Add 210ul of [PCR product and PB buffer] to the column with silica membrane, and centrifuge at 130k rpm for 1 min.
2. Add 750ul of PE buffer and centrifuge at 130k rpm for 1 min.
3. Centrifuged again at 130k rpm for 1 min.
4. Add 10ul of DW, and centrifuge at 130k rpm for 1min to elute.
measured concentration by nanodrop→-30℃

How to make acrylamide gel

10cm×10cm minigel

1.mix the following components.

40% acrylamide	2mL
5x TBE	1mL
DW	7mL

2. add **80ul of APS(10%) and 8uL of TEMED** **in this order.**
3.Pour into the mold immediately after step2.

In this case, the Electrophoresis condition is **100V 60min**.

Miniprep

1. Harvest 1–5ml (high-copy-number plasmid) or 10ml (low-copy-number plasmid) of bacterial culture by **centrifugation for 5 minutes at 10,000 x g i**n a tabletop centrifuge. Pour off the supernatant and blot the inverted tube on a paper towel to remove excess media.

2. **Add 250µl of Cell Resuspension Solution** and completely **resuspend** the cell pellet by vortexing or pipetting. It is essential to thoroughly resuspend the cells. If they are not already in a microcentrifuge tube, transfer the resuspended cells to a sterile 1.5ml microcentrifuge tube(s). Note: To prevent shearing of chromosomal DNA, do not vortex after Step 2. Mix only by inverting

the tubes.

3. **Add 250µl of Cell Lysis Solution** and mix by inverting the tube 4 times (**do not vortex**). Incubate until the cell suspension clears (approximately 1–5 minutes). Note: It is important to observe partial clearing of the lysate before proceeding to addition of the Alkaline Protease Solution (Step 4); however, do not incubate for longer than 5 minutes.

4. **Add 10µl of Alkaline Protease Solution** and mix by inverting the tube 4 times. **Incubate for 5 minutes at room temperature.** Alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA. Do not exceed 5 minutes of incubation with Alkaline Protease Solution at Step 4, as nicking of the plasmid DNA may occur.

5. **Add 350µl of Neutralization Solution** and immediately mix by inverting the tube 4 times (do not vortex).

6. **Centrifuge the bacterial lysate at maximum speed (around 14,000 × g) in a microcentrifuge for 10 minutes at room temperature.**

Prepare plasmid DNA purification units by inserting one Spin Column into one 2ml Collection Tube for each sample.

1. Transfer the cleared lysate (approximately 850µl, Section 3.B, Step 6) to the prepared Spin Column by decanting. Avoid disturbing or transferring any of the white precipitate with the supernatant. Note: If the white precipitate is accidentally transferred to the Spin Column, pour the Spin Column contents back into a sterile 1.5ml microcentrifuge tube and centrifuge for another 5–10 minutes at maximum speed. Transfer the resulting supernatant into the same Spin Column that was used initially for this sample. The Spin Column can be reused but only for this sample.

2. **Centrifuge the supernatant at maximum speed in a microcentrifuge for 1 minute at room temperature.** Remove the Spin Column from the tube and discard the flow through from the Collection Tube. Reinsert the Spin Column into the Collection Tube.

3. **Add 750µl of Column Wash Solution**, previously diluted with 95% ethanol, to the Spin Column.

4. **Centrifuge at maximum speed in a microcentrifuge for 1 minute at room temperature.** Remove the Spin Column from the tube and discard the flow through. Reinsert the Spin Column into the Collection Tube.

5. **Repeat the wash procedure using 250µl of Column Wash Solution.**

6. **Centrifuge at maximum speed in a microcentrifuge for 2 minutes at room temperature.**

7. Transfer the Spin Column to a new, sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the Column Wash Solution with the Spin Column. If the Spin Column has Column Wash Solution associated with it, centrifuge again for 1 minute at maximum speed.

8. Transfer the Spin Column to a new, sterile 1.5ml microcentrifuge tube.

9. **Elute the plasmid DNA by adding 100µl of Nuclease-Free Water to the Spin Column.** Centrifuge at maximum speed for 1 minute at room temperature in a microcentrifuge.

10. After eluting the DNA, remove the assembly from the 1.5ml microcentrifuge tube and discard the Spin Column.

11. DNA is stable in water without addition of a buffer if stored at –20°C or below. DNA is stable

at 4°C in the TE buffer. To store the DNA in TE buffer, add 11µl of 10X TE buffer to the 100µl of eluted DNA. Do not add a TE buffer if the DNA is to be used for automated fluorescent sequencing.

12. Cap the microcentrifuge tube and store the purified plasmid DNA at –20°C or below

Infront Assembly protocol

0.
set the thermal cycler program as follows;
This helps you immediately conduct step2 after step1.

℃	time
37	2min
37	5min
65	5min

1.
Mix the fragments (about 10-30 ng/microL) and 2x IA KKT (Infront Assembly 2x mix) on ice and transfer to the PCR tube.

ex):

components	conc.	volume (uL)
insert	10ng/uL	1
linearized backbone	20ng/uL	1
2× IA KKT	2x	2

2. set the reaction mix on a thermal cycler, and after this thaw DH5α(from liquid nitrogen) on ice.
3.add 2.5uL of the reaction mix to 10uL of DH5α.
4.incubate on ice for 20 minutes.
5.heat-shock at 42℃ for 45℃
6.incubate on ice for 1 minute.
7.add 100uL of SOC. (10x volume of competent cells)
8.incubate at 37℃.
9.spread onto LB plate(including proper antibiotics)
10.incubate at 37℃.

0909

oligo no template PCR

samples:

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
oligo_A	f042	71	f043	71	71	137

oligo_B_1	f058	62.8	f059	62.8	63	116
oligo_C	f062	65	f063	65	65	129
oligo_ParS	f032	77.9	f033	77.9	78	112
oligo_B_2	f060	65.9	f061	65.9	66	116
oligo_D	f055	69.3	f056	69.3	69	114

PCR program:

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	12 cycle
extension	68	30sec	↓
hold	22	∞	

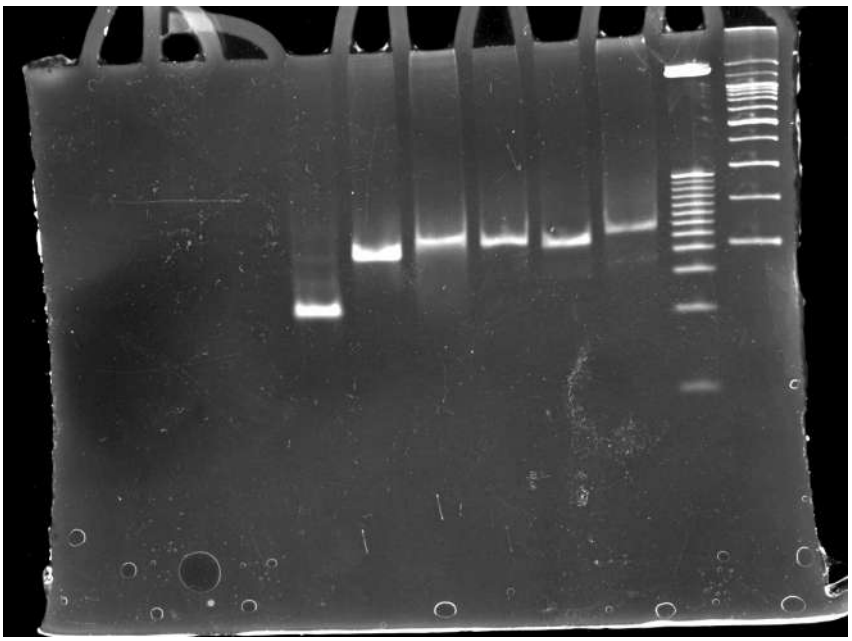
reaction composition:

components	conc.	volume (uL)	final conc.
distilled water		11.6	
5x PCR Buffer		4.0	1x
dNTPs	2.5mM	1.6	0.5mM each
F primer	10uM	1.2	0.6uM
R primer	10uM	1.2	0.6uM
Prime STAR GXL		0.4	
total		20	

*no template

0910

Elecrophoresis
100V 60min



from right to left: oligo_A / oligo_B_1 / oligo_C / oligo_ParS / oligo_B_2 / oligo_D

→staining by EtBr for 5min
→cut the gel / store in 0.3M NaCl

0911

column purification :
1. Add 210ul of [PCR product and PB buffer] to the column with silica membrane, and centrifuge at 130k rpm for 1 min.
2. Add 750ul of PE buffer and centrifuge at 130k rpm for 1 min.
3. Centrifuged again at 130k rpm for 1 min.
4. Add 10ul of DW, and centrifuge at 130k rpm for 1min to elute.
measured concentration by nanodrop→-30℃

サンプル名	核酸(ng/uL)
oligo_A	44.2ng/uL
oligo_B1	35.0ng/uL
oligoC	29.2ng/uL
oligo_ParS	49.1ng/uL

oligo_B2	63.0ng/uL

gblock PCR

template		F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
CFPsopB_1	gBlock PCR	f020	55.9	f021	53.3	55	977
CFPsopB_2	gBlock PCR	f001	60.1	f019	62	60	851
CFPparB_1	gBlock PCR	f029	58	f030	60.5	60	903
CFPparB_2	gBlock PCR	f031	60.5	f002	60.6	60	921
iRFP	gBlock PCR	f001	60.1	f016	56.9	60	1009

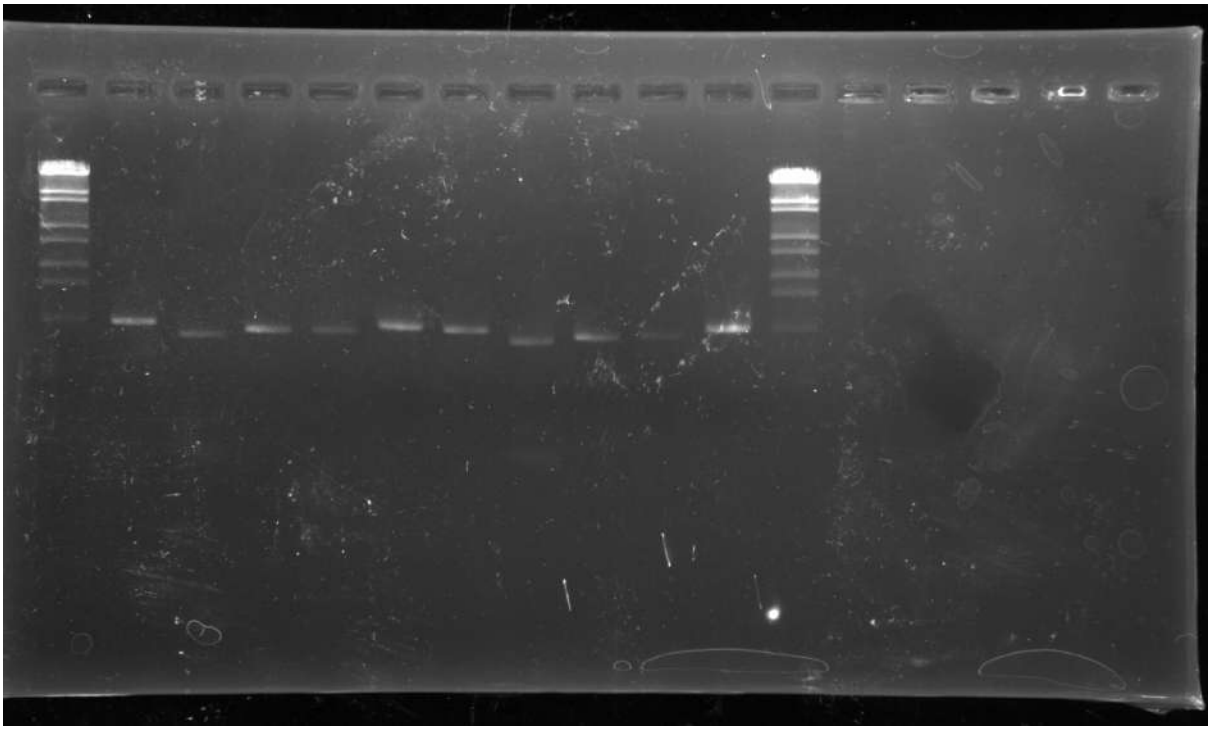
template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	11.2ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	30sec	16 cycle
extension	68	30sec	↓
hold	22	∞	

↑mistake

steps	℃	time	

predenature			
denature	98	10sec	↑
annealing	60	30sec	16 cycle
extension	68	1min	↓
hold	22	∞	



looks good!

after column purification, measured the dsDNA concentration by nanodrop

サンプル名	核酸(ng/uL)	A260/A280	A260/A230
CFPsopB_1	47.1	1.89	1.96
CFPsopB_2	20.5	1.91	1.49
CFPparB_1	18.0	1.96	1.80
CFPparB_2	7.6	2.29	1.45
iRFP	44.2	1.96	2.03

Nco1

restriction enzyme reaction → plasmid template PCR

pSB4K5-mRFP(56.1ng/ul)	2ul
bufferK	1ul
Nco1	0.5ul
DW	6.5ul
total	10ul

pSB1A3-mRFP(75.7ng/ul)	2ul
bufferK	1ul
Nco1	0.5ul
DW	6.5ul
total	10ul

pSB4K5-mRFP(147.4ng/ul)	1ul
bufferK	1ul
Nco1	0.5ul
DW	7.5ul
total	10ul

incubate at 37℃ for 1hr

PCR(template: plasmid)

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul

Total	20uL

PCR program:

insert

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

backbone

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	3.5min	↓
hold	16	∞	

template			F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
A	sfGFP	BioBrick PCR	f017	53	f018	51.8	52	789
C	DT_1	BioBrick PCR	f026	54.9	f024	55.1	55	189
D	DT_1	BioBrick PCR	f026	54.9	f024	55.1	55	189
B	DT_2	BioBrick PCR	f024	55.1	f025	57.1	56	189
D	DT_3	BioBrick PCR	f024	55.1	f046	62.1	59	189
A	pSB1A3	BioBrick PCR	f004	59.9	f003	60.1	60	2155
C	pSB3C5	BioBrick PCR	f004	59.9	f003	60.1	60	2738
D	pSB3C5	BioBrick PCR	f004	59.9	f003	60.1	60	2738

B	pSB4K5	BioBrick PCR	f004	59.9	f003	60.1	60	3419
C	TetR1	BioBrick PCR	f027	70.8	f045	68.5	68	660
B	cI_1	BioBrick PCR	f057	66.6	f023	70.1	68	750
D	cI_2	BioBrick PCR	f044→f107	66.6	f023	70.1	68	780
D	TetR2	BioBrick PCR	f027	70.8	f082	68.5	69	690

0913

gel purification
(**oligo - D**)
1.centrifuge(14000 rpm 15min)
2. Remove supernatant
3. Add 150ul of 85% EtOH
4.centrifuge(14000 rpm 3min)
5. Remove supernatant
6. centrifuge (Flash)
7. Remove the supernatant
8. Elute with 15ul of DW

09132021(Tajima)

PCR(template: plasmid)

retry【1st time】

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

PCR program:
insert

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	1min	↓
hold	16	∞	

backbone

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	3.5min	↓
hold	16	∞	

retry【2nd time】

use backbone digested with HincII to linearize.

Fast Digest HincII	1uL
10X M Buffer	2uL
MilliQ	15.5uL
plasmid*	2uL
total	20uL

incubated at 37℃ for 30min, then at 65℃ for 5min to heat-inactivate.
After the digestion, the sample was directly used as a PCR template.

*pSB4K5-mRFP(56.1ng/ul), pSB1A3-mRFP(75.7ng/ul), pSB4K5-mRFP(147.4ng/ul)

template**	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul

primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

**pSB4K5-mRFP(3.7ng/ul), pSB1A3-mRFP(3.8ng/ul), pSB4K5-mRFP(3.7ng/ul)

PCR program:

insert

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	35 cycle
extension	68	1min	↓
hold	16	∞	

backbone

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	35 cycle
extension	68	3.5min	↓
hold	16	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
A	pSB1A3	BioBrick PCR	f004	59.9	f003	60.1	60	2155
C	pSB3C5	BioBrick PCR	f004	59.9	f003	60.1	60	2738
D	pSB3C5	BioBrick PCR	f004	59.9	f003	60.1	60	2738
B	pSB4K5	BioBrick PCR	f004	59.9	f003	60.1	60	3419
B	cI_1	BioBrick PCR	f057	66.6	f023	70.1	68	750
D	cI_2	BioBrick PCR	f044→f107	66.6	f023	70.1	68	780
D	TetR2	BioBrick	f027	70.8	f082	68.5	69	690

		PCR						

after column purification, measured the dsDNA concentration by nanodrop

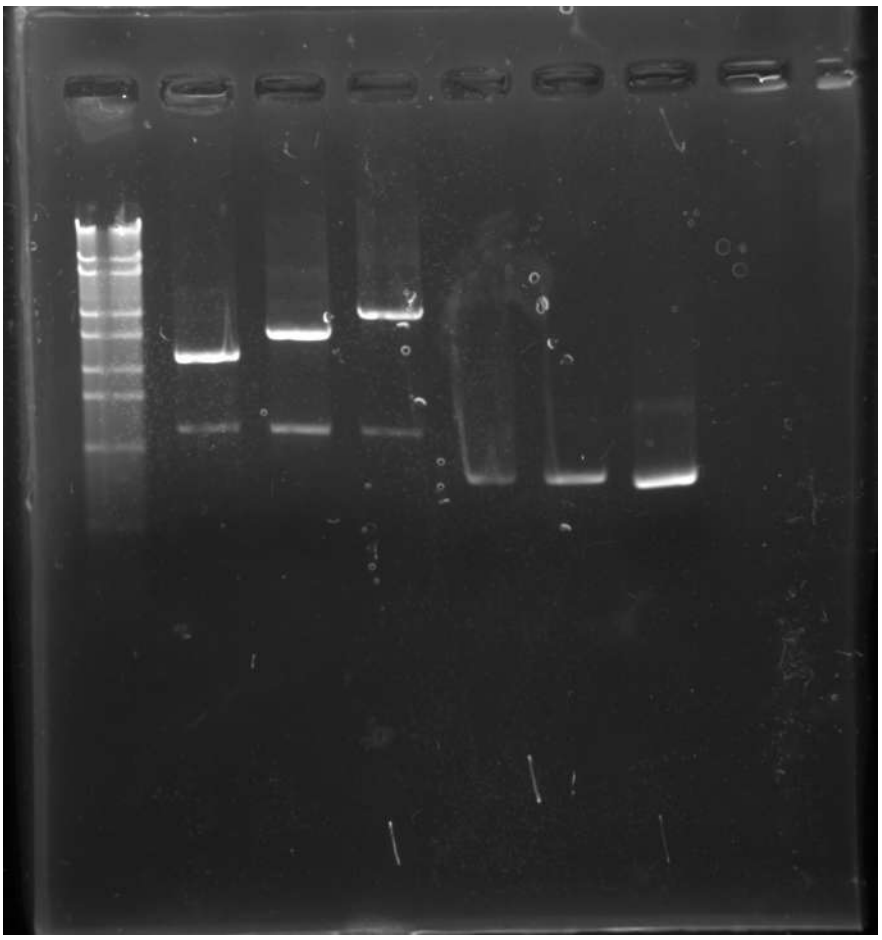
サンプル名	核酸(ng/uL)	A260/A280	A260/A230
sfGFP	99.4	1.89	2.21
DT_1	35.3	1.88	1.94
DT_2	49.5	1.88	2.05
DT_3	54.0	1.90	2.20
pSB1A3	93.2	1.87	2.17
pSB3C5	268	1.90	1.97
pSB4K5	94.6	1.88	2.17
TetR1	66.4	1.88	2.16
cI_1	227.3	1.89	2.34
cI_2	208.9	1.88	2.29
TetR2	224.8	1.88	2.36

Electrophoresis

100V 20min / 1% agarose TAE gel

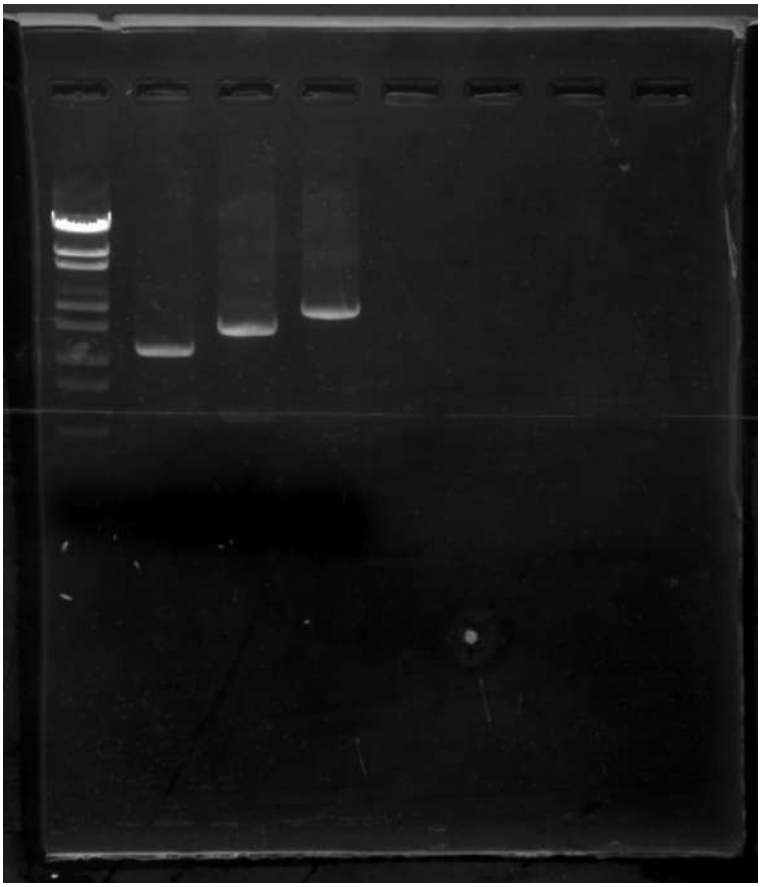
[1st one]

(from left to right: pSB1A3, pSB3C5, pSB4K5, cI-1, c1-2, TetR-2)



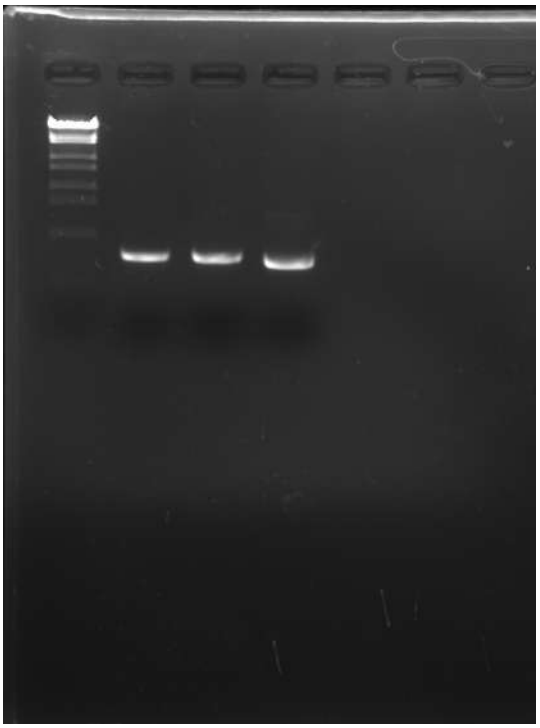
the bands look good!

[2nd one]
backbone
(from left to right: pSB1A3, pSB3C5, pSB4K5)



insert

(from left to right: cI-1, c1-2, TetR-2)



The bands look good , so we conducted Dpn1 treatment.

(added 1uL of Dpn1 to each 18uL of samples.)

BioBrick PCR (for sopC)

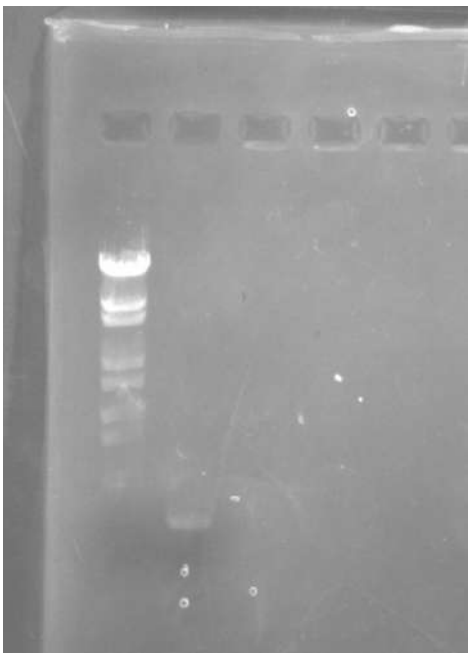
template(1ng/uL)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
pMK755	f049	55	f050	55.5	55	580

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	40sec	↓
hold	16	∞	

after column purification, measured the dsDNA concentration by nanodrop

サンプル名	核酸(ng/uL)	A260/A280	A260/A230
sopC	29.4	1.90	2.32



The band looks good.

OE-PCR_1

plasmidA
pSB1A3-mRFP:→backbone
gB_iRFP
oligo_A
sfGFP

plasmidB
pSB4K5-mRFP:→backbone
gB_lp-ecfp-sopB_2
gB_lp-ecfp-sopB_1
oligo_B_1
oligo_B_2
cI-1
DT2
sopC

plasmidC
pSB3C5-mRFP:→backbone
oligo_ParS
DT1
TetR1
oligoC
gB_lp-ecfp-parB_1
gB_lp-ecfp-parB_2

plasmidD
pSB4K5-mRFP:→backbone

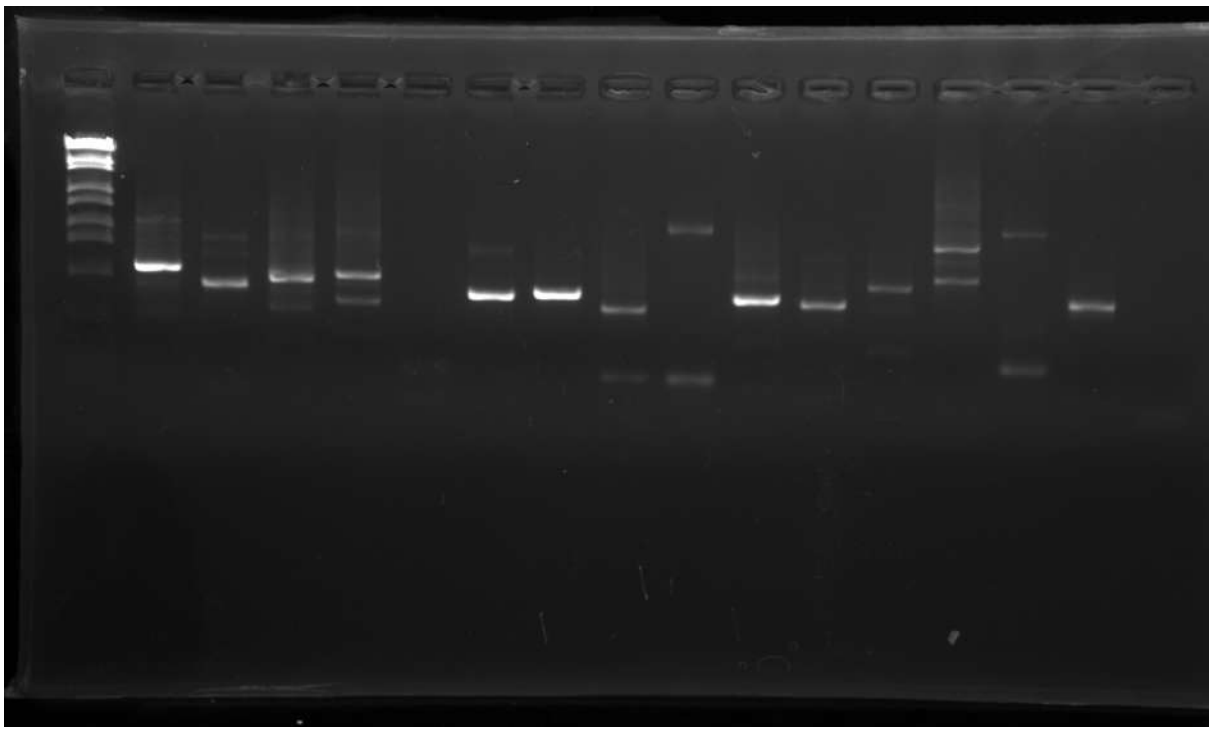
oligo_ParS
DT1
TetR2
oligo_D
cI-2
DT3
gB_lp-ecfp-parB_1
gB_lp-ecfp-parB_2

No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
1	A	iRFP	oligo_A	f001	60.1	f043	71		1116
2	A	oligo_A	sfGFP	f042	71	f018	51.8		896
3	B	gB_lp-ecfp-sopB_2	gB_lp-ecfp-sopB_1	f001	60.1	f021	53.3		1798
4	B	gB_lp-ecfp-sopB_1	oligo_B_1	f020	55.9	f059	62.8		1063
5	B	oligo_B_1	oligo_B_2	f058	62.8	f061	65.9		192
6	B	oligo_B_2	cI-1	f060	65.9	f023	70.1		836
7	B	cI-1	DT2	f057	66.6	f025	57.1		879
8	B	DT2	sopC	f024	55.1	f002	60.6		700
9	C	oligo_parS	DT_1	f001	60.1	f024	55.1		271
10	C	DT_1	TetR1	f026	54.9	f045	68.5		819
11	C	TetR1	oligo_C	f027	70.8	f063	65		759
12	C	oligo_C	CFPparB_1	f062	65	f030	60.5		1002
1	C	CFPpar	CFPpar	f029	58	f002	60.6		1794

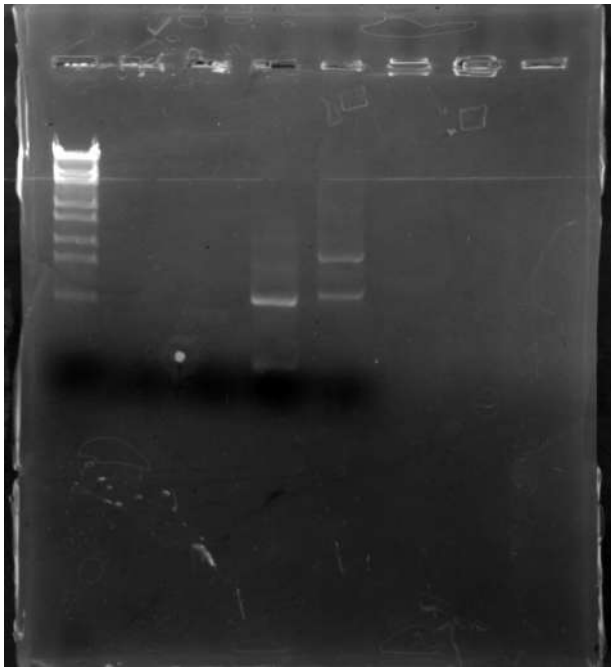
3		B_1	B_2						
14	D	oligo_ParS	DT_1	f032	77.9	f024	55.1		271
15	D	DT_1	TetR2	f026	54.9	f082	68.5		849
16	D	TetR2	oligo_D	f027	70.8	f056	69.3		774
17	D	oligo_D	cI_2	f055	69.3	f023	70.1		864
18	D	cI_2	DT_3	f044→f107	66.6	f046	62.1		939
19	D	DT_3	CFPparB_1	f024	55.1	f030	60.5		1062
20	D	CFPparB_1	CFPparB_2	f029	58	f002	60.6		1794

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

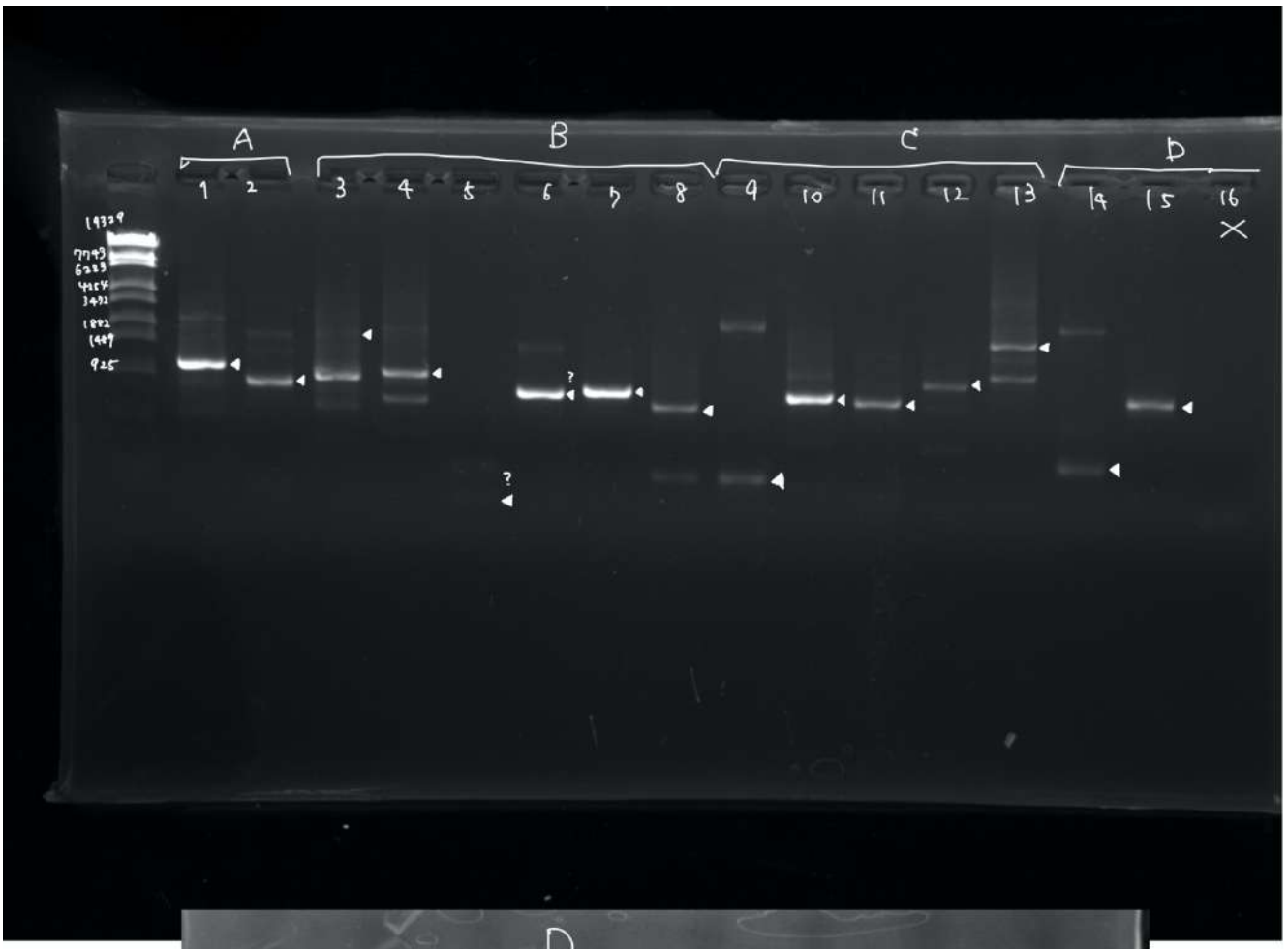
Electrophoresis: 100V 20min / 1% agarose TAE gel

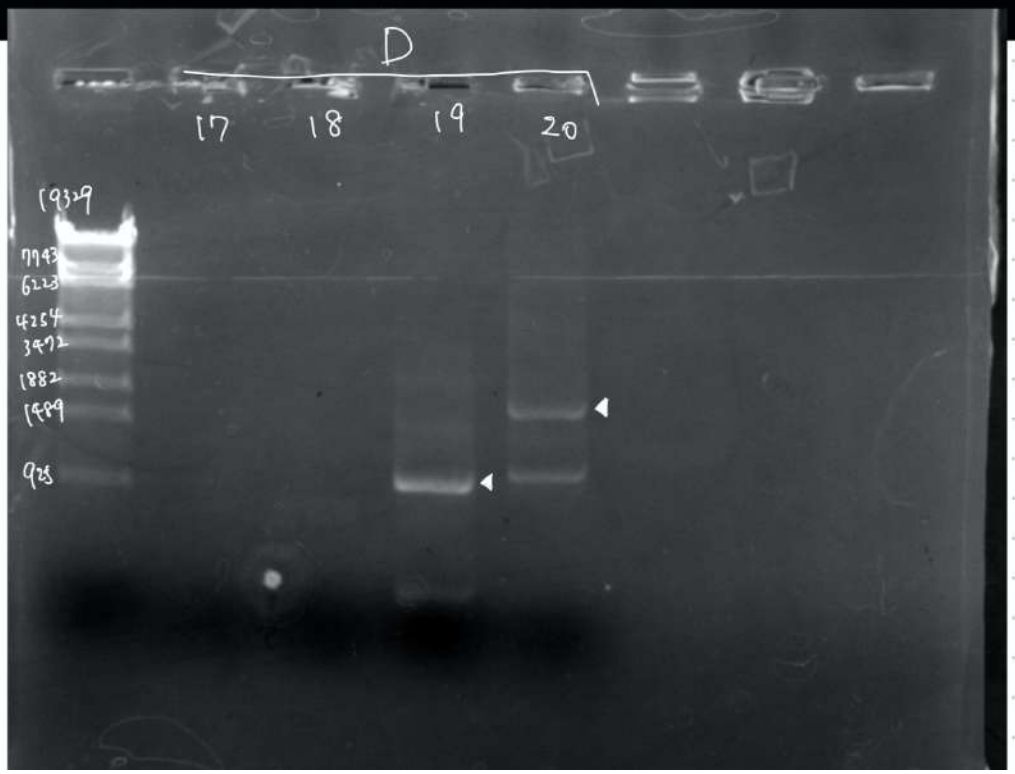


(from left to right: 1-16
expected size: 1116, 896, 1798, 1063, 192, 836, 879, 700, 271, 819, 759, 1002, 1794, 271, 849, 774)



(from left to right: 17-20
expected size: 864, 939, 1062, 1794)





A: iRFP,1,backbone
B: 4,6,8, backbone
C:9,11,13,backbone

0915

for 1,4,6,8,9,11,13
→column purification

サンプル名	核酸(ng/uL)	A260/A280	A260/A230
1	155.6	1.90	2.23
4	83.7	1.90	2.18
6	94.2	1.87	2.31
8	42.4	1.98	2.49
9	62.0	1.91	2.26
11	65.9	1.90	2.30
13	123.6	1.90	2.11

looks great! 🐈🐈🐈🐈🐈🐈🐈🐈🐈🐈🐈🐈

for all samples
→by LMP gel, conduct gel purification

サンプル名	核酸(ng/uL)
1	13.6
2	15.2
3	5.2
4	2.24
6	7.94
7	35.2
8	2.18
9	3.04
10	67.0

11	5.80
12	14.8
13	12.6
14	12.0
15	9.46
19	26.0
20	3.70

gel purification
1, Weigh the gel (define the weight of the Eppendorf tube as 0).
2. Add 4x of TE or water.
3. Melt the gel at 65°C for 20 minutes. Vortex several times during this time.
4. Add an equal amount of TE-saturated phenol.
*** For LMP agarose gels, do not use chloroform treatment!!!)**
5.centrifuge at max speed for 10minutes
6. The liquid is divided into three layers from the top: a transparent layer, a white layer, and a yellow layer, so take only the transparent layer.
7. centrifuge at max speed for 5minutes again ←important!!!!
8. To concentrate the water layer, add 2-butanol 500uL at a time, vortex, and centrifuge to remove only the water-absorbed butanol from the top layer→ Repeat this about 3 times until the water DNA's volume becomes 200uL or so.
9. Add 1/10 of the sample volume of NaOAce.
10. Add glycogen 2uL and 3× 100% alcohol for each sample
→→→→
next day
1.centrifuge(14000 rpm 15min)
2. Remove supernatant
3. Add 150ul of 85% EtOH
4.centrifuge(14000 rpm 3min)
5. Remove supernatant
6. centrifuge (Flash)
7. Remove the supernatant
8. Elute with 15ul of DW

Infront Assembly

Infront Assembly protocol

0.
set the thermal cycler program as follows;
This helps you immediately conduct step2 after step1.

℃	time

37	hold
37	5min
65	5min
4	hold

1.
Mix the fragments (about 10-30 ng/microL) and 2x IA KKT (Infront Assembly 2x mix) on ice and transfer it to the PCR tube.

1. plasmid A

components	conc.	volume (uL)
OE-PCR 1	10ng/uL	1
iRFP	10ng/uL	1
pSB1A3	20ng/uL	1
2× IA KKT	2x	3

2. plasmid A control

components	conc.	volume (uL)
DW		1
iRFP	10ng/uL	1
pSB1A3	20ng/uL	1
2× IA KKT	2x	3

3. plasmid B

components	conc.	volume (uL)
OE-PCR 4	10ng/uL	1
OE-PCR 6	10ng/uL	1
OE-PCR 8	10ng/uL	1
pSB4K5	20ng/uL	1
2× IA KKT	2x	4

4. plasmid B control

components	conc.	volume (uL)
OE-PCR 4	10ng/uL	1
OE-PCR 6	10ng/uL	1
DW		1
pSB4K5	20ng/uL	1
2× IA KKT	2x	4

5. plasmid C

components	conc.	volume (uL)

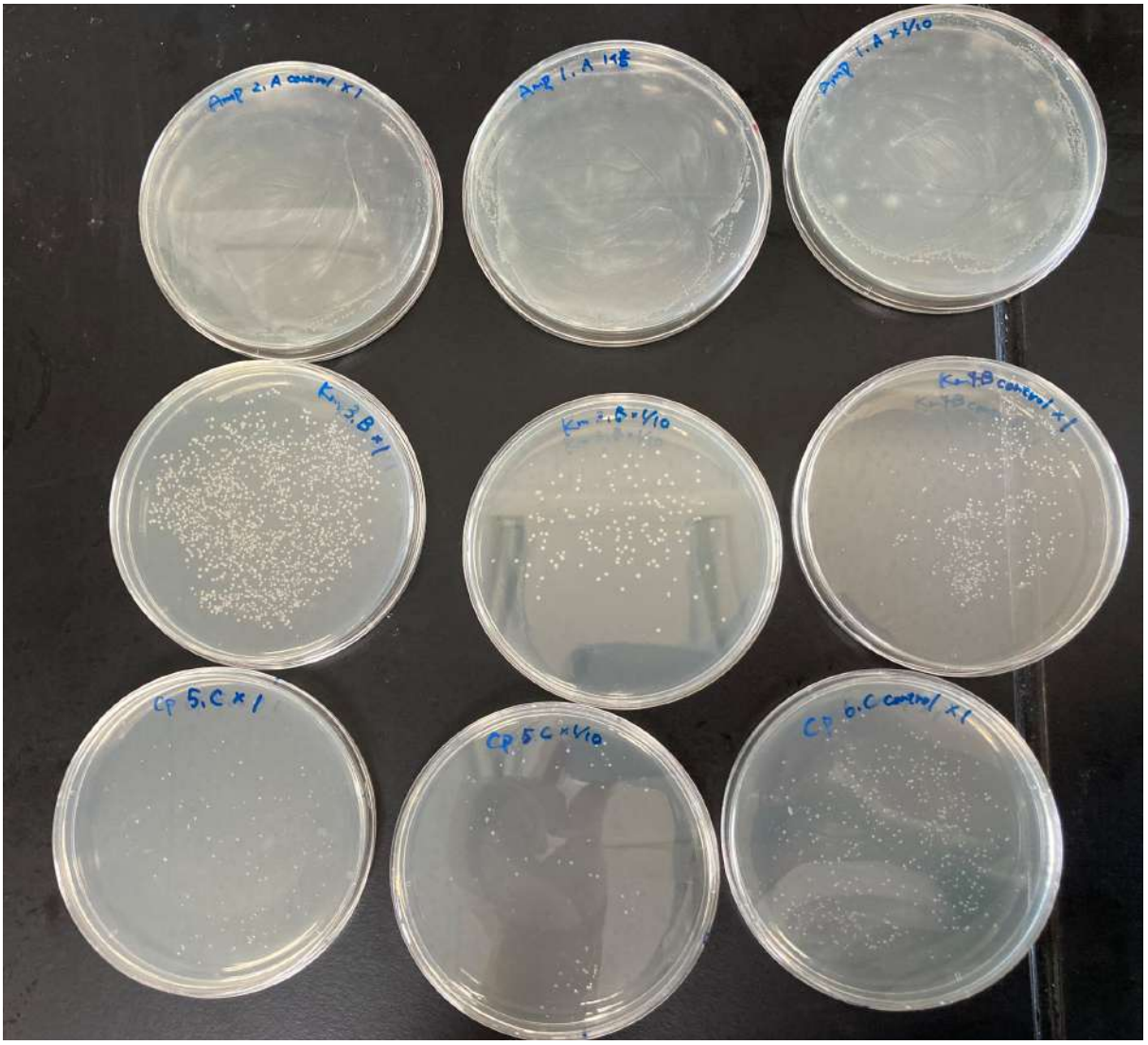
OE-PCR 9	10ng/uL	1
OE-PCR 11	10ng/uL	1
OE-PCR 13	10ng/uL	1
pSB3C5	20ng/uL	1
2× IA KKT	2x	4

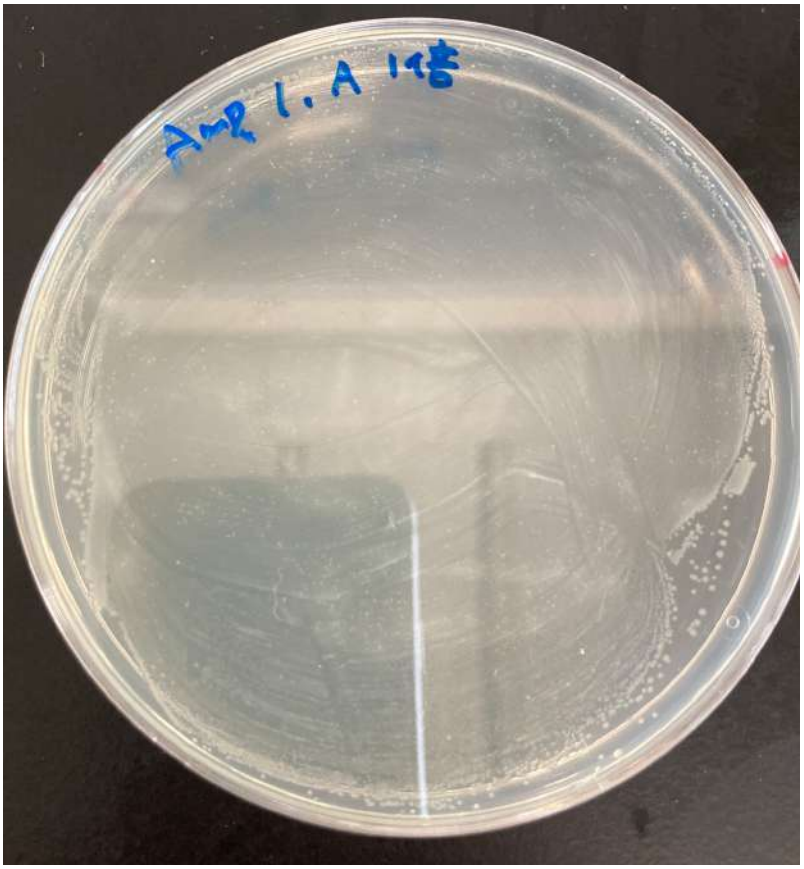
6. plasmid C control

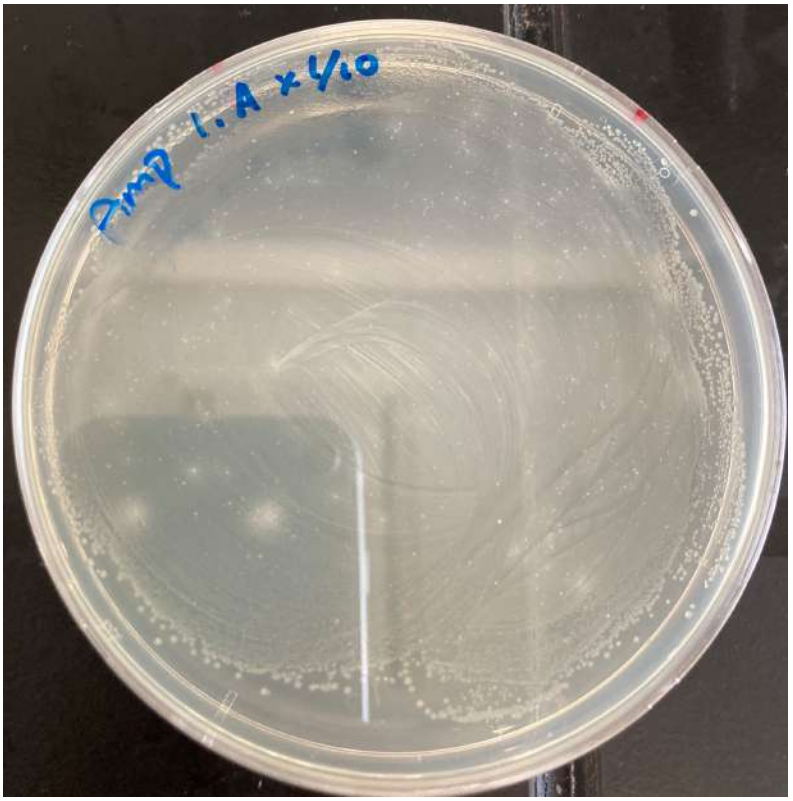
components	conc.	volume (uL)
OE-PCR 9	10ng/uL	1
OE-PCR 11	10ng/uL	1
DW		1
pSB3C5	20ng/uL	1
2× IA KKT	2x	4

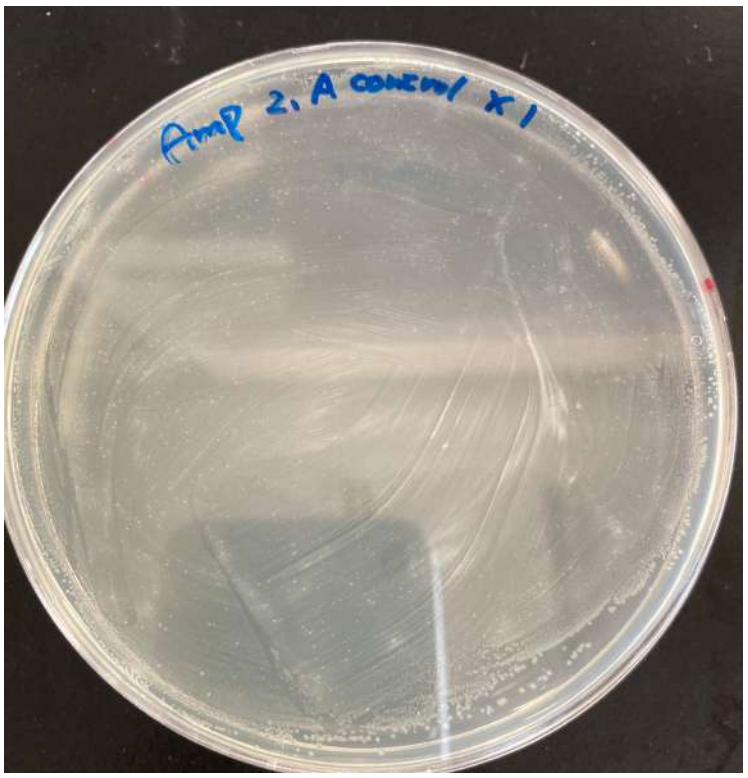
2. set the reaction mix on a thermal cycler, and after this thaw DH5α(from liquid nitrogen) on ice.
3.add 4uL of the reaction mix to 40uL of DH5α.
4.incubate on ice for 20 minutes.
5.heat-shock at 42℃ for 45 sec
6.incubate on ice for 1 minute.
7.add 400uL of SOC. (10x volume of competent cells)
8.incubate at 37℃ for 1h.
9.spread it onto LB plate(including proper antibiotics)
10.incubate at 37℃. o/n

0916

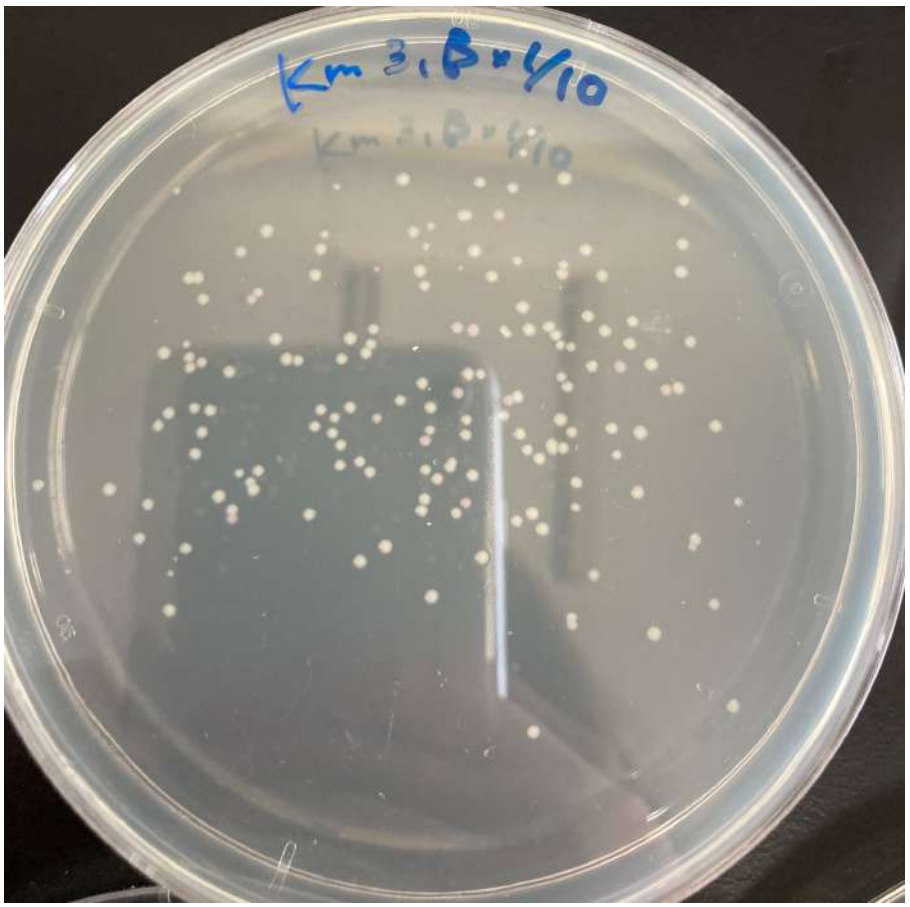

Amp 2.A control x1
Amp 1.A 1/5
Amp 1.A x1/10
Km 3.B x1
Km 2.B x1/10
Km 1.B control x1
Cp 5.C x1
Cp 5.C x1/10
Cp 6.C control x1

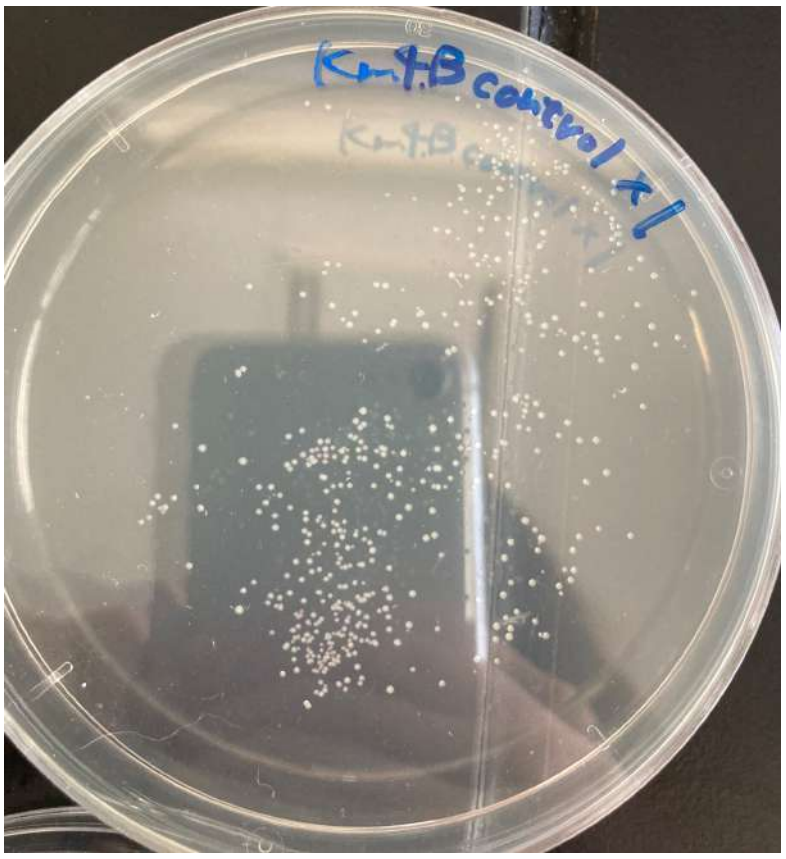

Amp 1.A 1µg

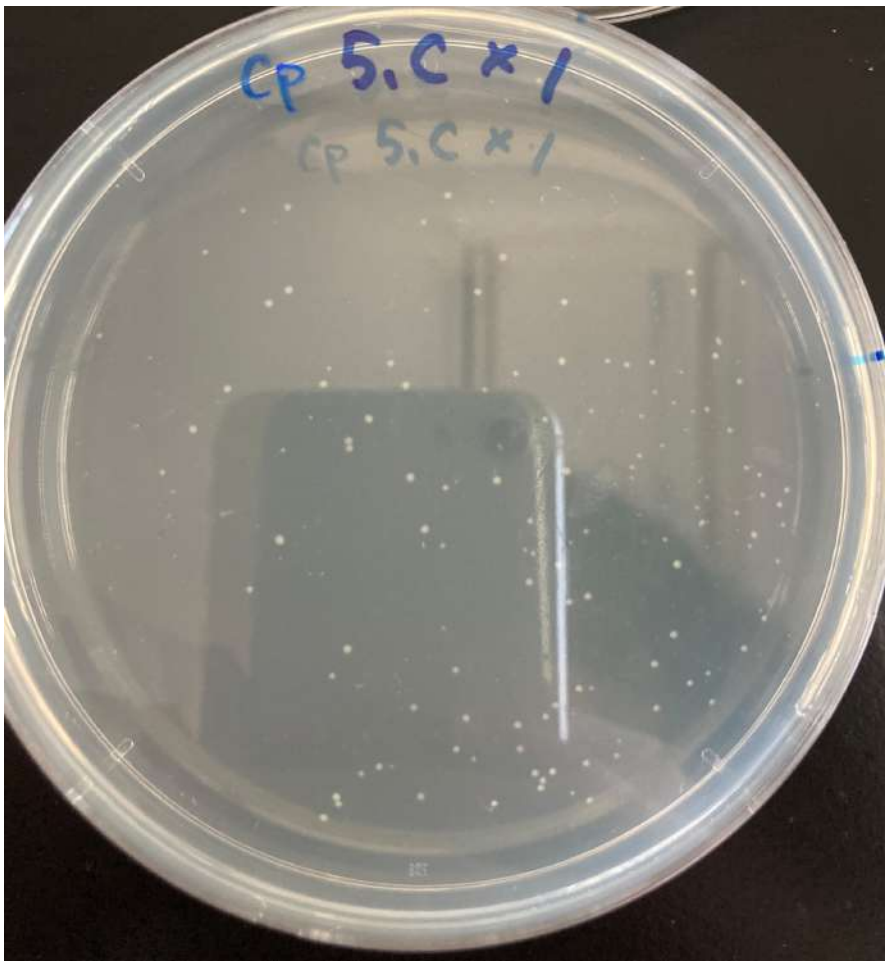

Amp 1.A x1/10

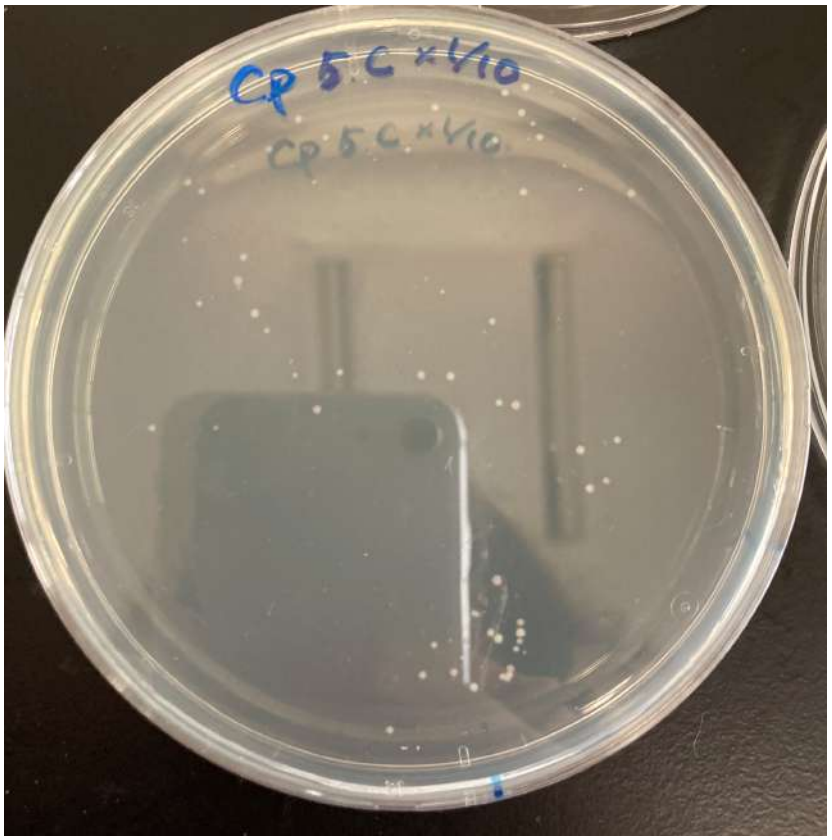

Amp 2. A control x1


Km 3. B x1


Km 3.B x 4/10


Km 1.B control x1


Cp 5.C x 1
Cp 5.C x 1


Cp 5.C x 1/10
Cp 5.C x 1/10

😢😢😢😢😢😢😢😢😢😢😢😢

PCR(new oligo_D)

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

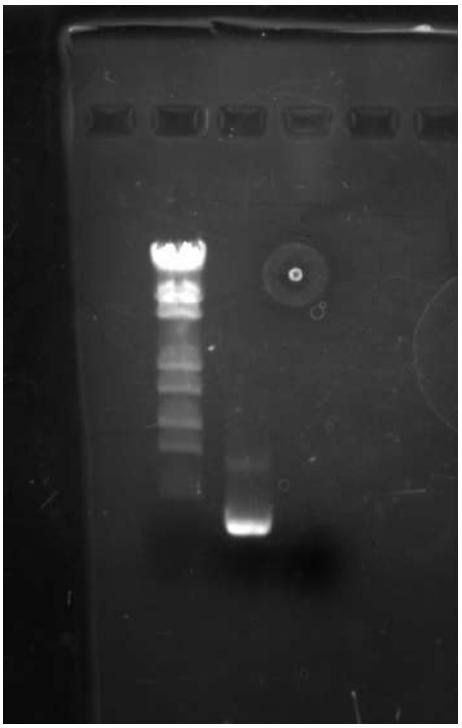
PCR program

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
TetR2	f027		f105			737
CI_2	f106		f023			827

Electrophoresis: 100V 20min / 1% agarose TAE gel

(from left to right: TetR-2, CI_2)



retry PCR(new oligo_D)

template*	*
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

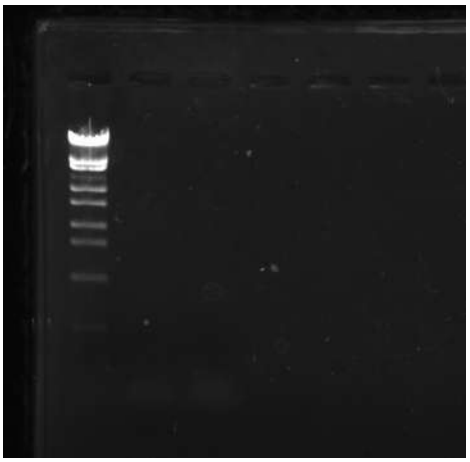
*OE-PCR15 : 9.46ng/ul 1.42ul
CI_2 : 1ng/ul 1ul

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
OE-PCR15	f026		f105			891
CI_2	f106		f023			827
f106	f106		f023			827

0917

Electrophoresis of PCR samples

1% LMP agarose gel / **50V 40 min**
from left to right CI-2(PCR sample by f106), CI-2(plasmid by f106)



no band...😢

Gel purification

サンプル名	核酸(ng/uL)
pSB1A3	12.1ng/uL
pSB3C5	24.5ng/uL
pSB4K5	18.3ng/uL
sfGFP	
gB sopB2	0.95
iRFP	1.37

concentration is not enough for yellow highlighted ones.
→retry from PCR
Electrophoresis of PCR samples
1.5% LMP agarose gel / **50V 40 min**

retry OE-PCR

No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
1	A	iRFP	oligo_A	f001	60.1	f043	71		1116
4	B	gB_lp-ecfp-sopB_1	oligo_B_1	f020	55.9	f059	62.8		1063
6	B	oligo_B_2	cI-1	f060	65.9	f023	70.1		836
8	B	DT2	sopC	f024	55.1	f002	60.6		700
9	C	oligo_parS	DT_1	f001	60.1	f024	55.1		271
11	C	TetR1	oligo_C	f027	70.8	f063	65		759
13	C	CFPparB_1	CFPparB_2	f029	58	f002	60.6		1794

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

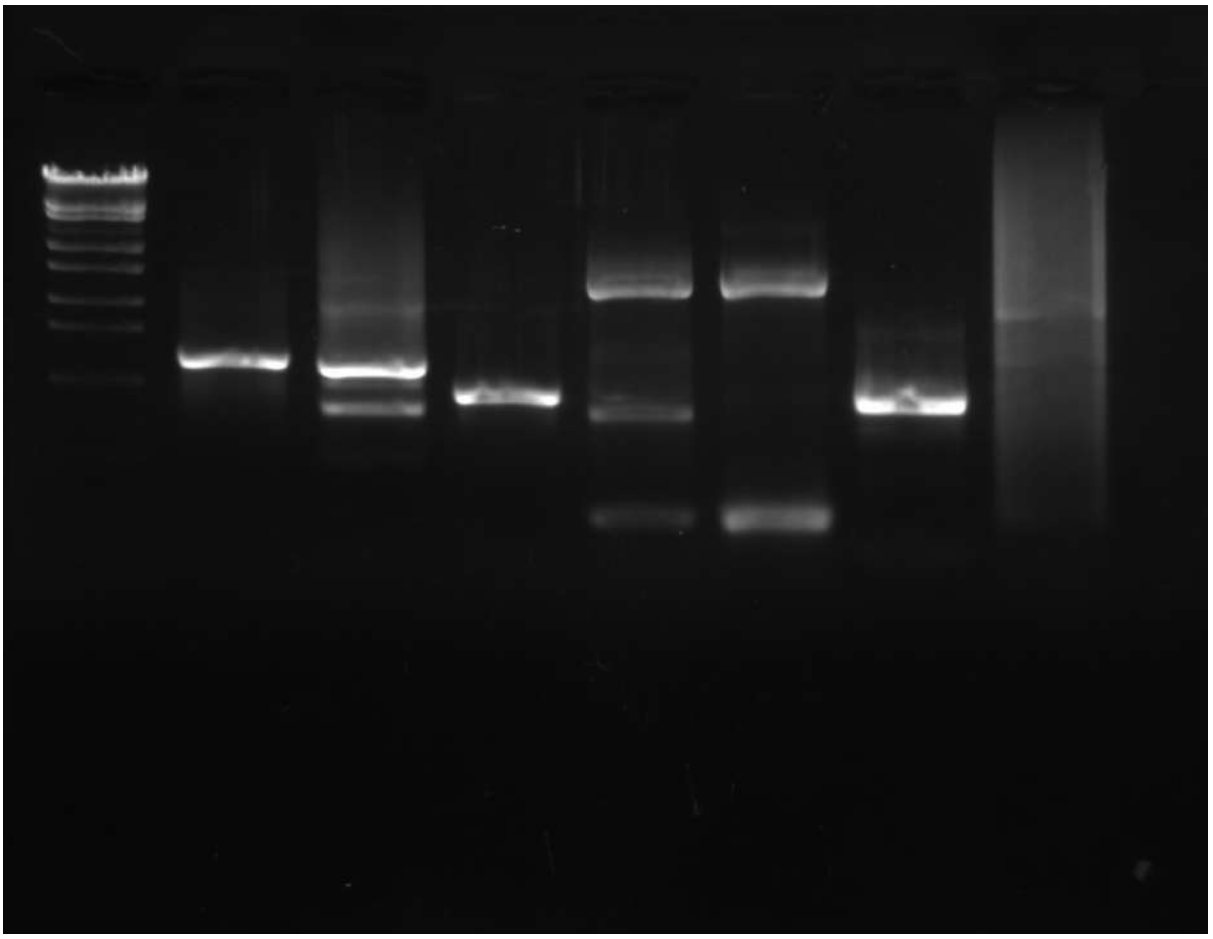
reaction composition:

components	conc.	volume (uL)	final conc.
distilled water		12.4	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	0.8	

PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

from left to right 1,4,6,8,9,11,13

expected size 1116, 1063, 836, 700, 271, 759, 1794



→retry PCR (4,8,9,13)

gel purification

サンプル名	核酸(ng/uL)
1	15.9
4	39.0
6	28.6

8	7.16
9	27.0
11	48.6
13	19.5

0918

retry infront assembly

using gel purification one

0.
set the thermal cycler program as follows;
This helps you immediately conduct step2 after step1.

℃	time
37	hold
37	5min
65	5min
4	hold

1.
Mix the fragments (about 10-30 ng/microL) and 2x IA KKT (Infront Assembly 2x mix) on ice and transfer it to the PCR tube.

1. plasmid A

components	conc.	volume (uL)
OE-PCR 1	10ng/uL	1
sfGFP	10ng/uL	1
linearized pSB1A3	20ng/uL	1
2× IA KKT	2x	3

2. plasmid A control 1

components	conc.	volume (uL)
DW		1
sfGFP	10ng/uL	1
linearized pSB1A3	20ng/uL	1

2× IA KKT	2x	3

3. plasmid B

components	conc.	volume (uL)
gBlock sopB2	10ng/uL	1
OE-PCR 4	10ng/uL	1
OE-PCR 6	10ng/uL	1
OE-PCR 8	10ng/uL	1
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	5

4. plasmid B control 1

components	conc.	volume (uL)
gBlock sopB2	10ng/uL	1
OE-PCR 4	10ng/uL	1
OE-PCR 6	10ng/uL	1
DW		1
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	5

5. plasmid C

components	conc.	volume (uL)
OE-PCR 9	10ng/uL	1
OE-PCR 11	10ng/uL	1
OE-PCR 13	10ng/uL	1
linearized pSB3C5	20ng/uL	1
2× IA KKT	2x	4

6. plasmid C control 1

components	conc.	volume (uL)
OE-PCR 9	10ng/uL	1
OE-PCR 11	10ng/uL	1
DW		1
linearized pSB3C5	20ng/uL	1

2× IA KKT	2x	4

2. set the reaction mix on a thermal cycler, and after this thaw DH5α(from liquid nitrogen) on ice.
3.add 4uL of the reaction mix to 40uL of DH5α.
4.incubate on ice for 20 minutes.
5.heat-shock at 42℃ for 45 sec
6.incubate on ice for 1 minute.
7.add 400uL of SOC. (10x volume of competent cells)
8.incubate at 37℃ for 1h.
9.spread it onto LB plate(including proper antibiotics)
10.incubate at 37℃. o/n

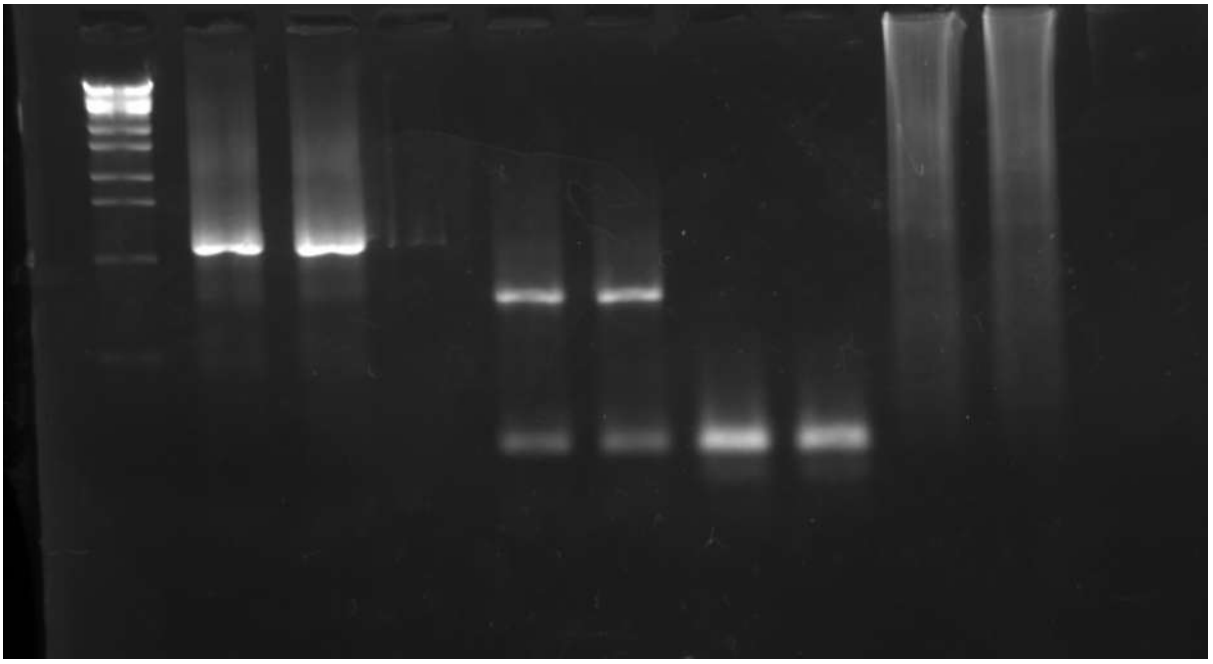
PCR(gel-purified OE-PCR 4,8,9,13)

template*	1ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

PCR program

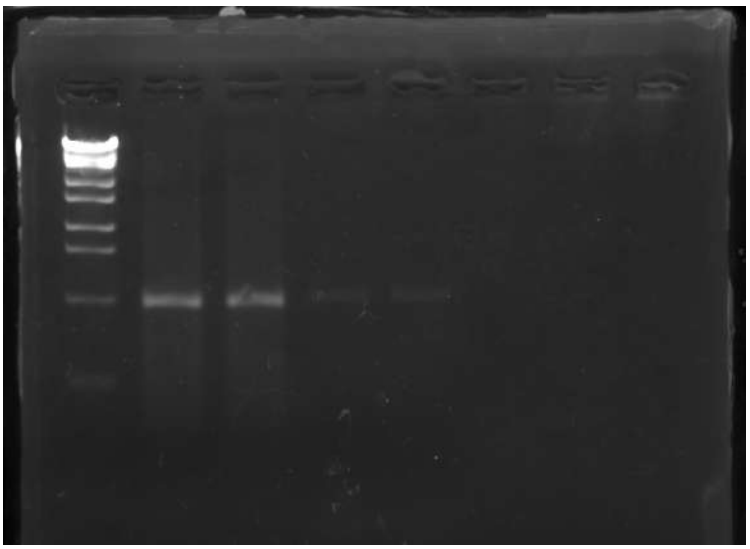
steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	25 cycle
extension	68	1min40sec	↓
hold	16	∞	

electrophoresis
50V 20min
from left to right 4,4,blank,8,8,9,9,13,13
expected size 1063, 700, 271, 1794



gel purification

No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
4	B	gB_lp-ecfp-sopB_1	oligo_B_1	f020	55.9	f059	62.8		1063
8	B	DT2	sopC	f024	55.1	f002	60.6		700
9	C	oligo_parS	DT_1	f001	60.1	f024	55.1		271
13	C	CFPparB_1	CFPparB_2	f029	58	f002	60.6		1794



gel purification

サンプル名	核酸(ng/uL)
4	19.5
8	10.8
9	19.6

0919

gblock PCR (parB_1,parB_2)

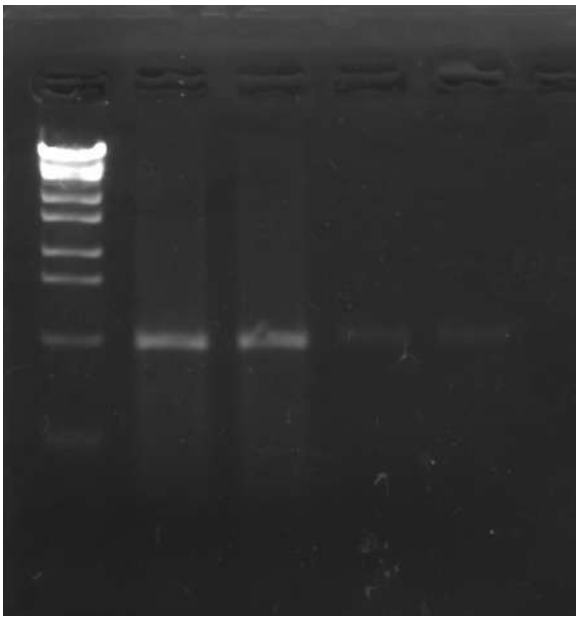
template		F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
CFPparB_1	gBlock PCR	f029	58	f030	60.5	60	903
CFPparB_2	gBlock PCR	f031	60.5	f002	60.6	60	921

template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul

5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	11.2ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	30sec	16 cycle
extension	68	1min	↓
hold	22	∞	

electrophoresis 50V 40min
1.5% LMP

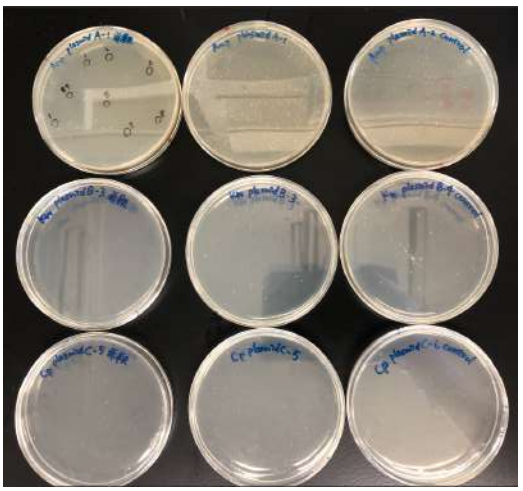


after gel purification, measured the dsDNA concentration

サンプル名	核酸(ng/uL)
CFPparB_1	4.78
CFPparB_2	0.840

low concentration...→retry gblock PCR

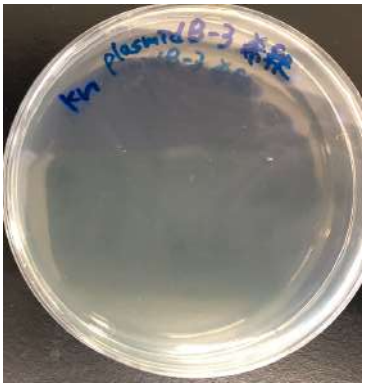
colony
after 17h



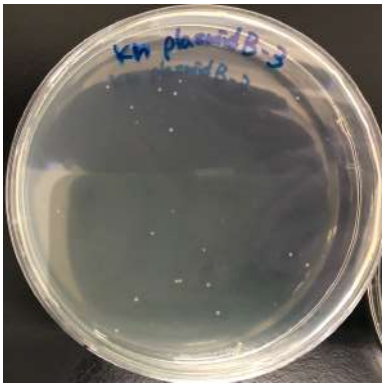

Amp plasmid A-1 森民
1
2
3
4
5
6
7
8

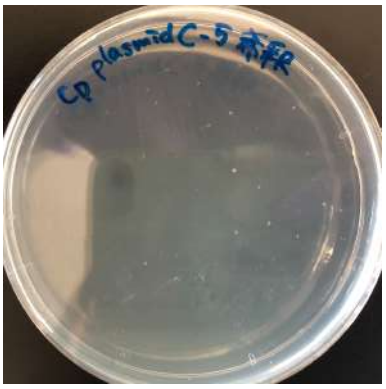

Amp plasmid A-1

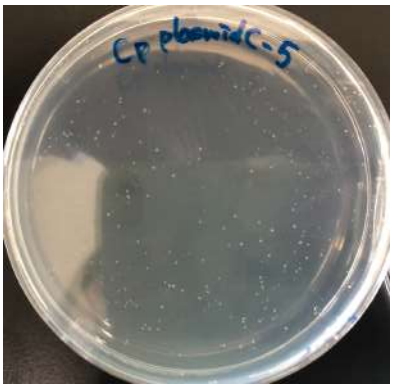

Amp plasmid A-2 control


Kn plasmid B-3 森民


Kn plasmid B-4 control


Kn plasmid B-3


Cp plasmid C-5 森民


Cp plasmid C-5

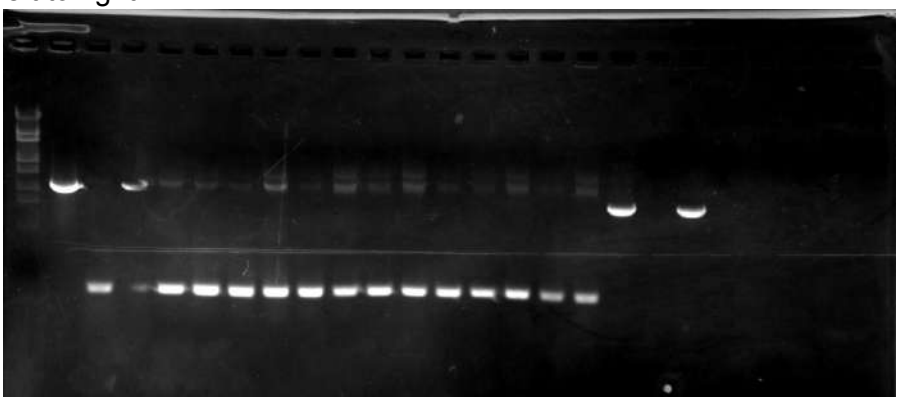

Cp plasmid C-6 control

colony PCR

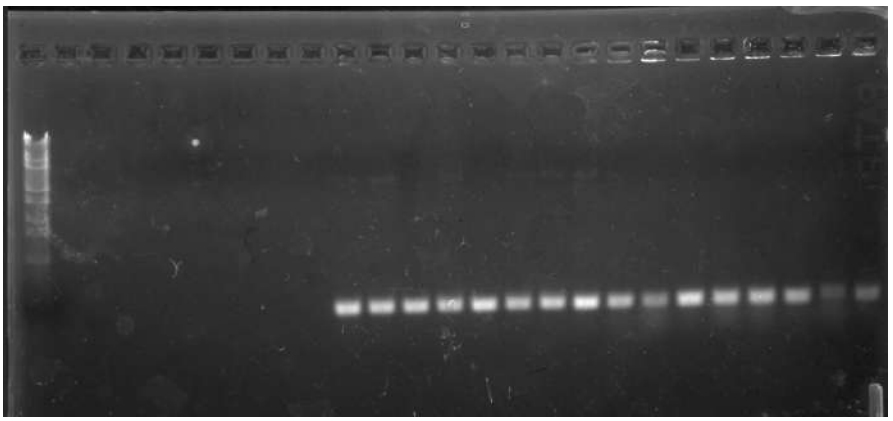
No.	plasmid	F primer ID	R primer ID	product size
1-8	A	f089	f090	2146
9-16	A control	f089	f090	
17-24	A	f089	f085	1620
25-32	A control	f089	f085	
33-40	B	f089	f090	3587
41-48	B control	f089	f090	
49-56	B	f083	f090	1198
57-64	B control	f083	f090	
65-72	C	f089	f090	3001
73-80	C control	f089	f090	
81-88	C	f062	f090	2007
89-96	C control	f062	f090	

f089 & f090 → whole length of inserts

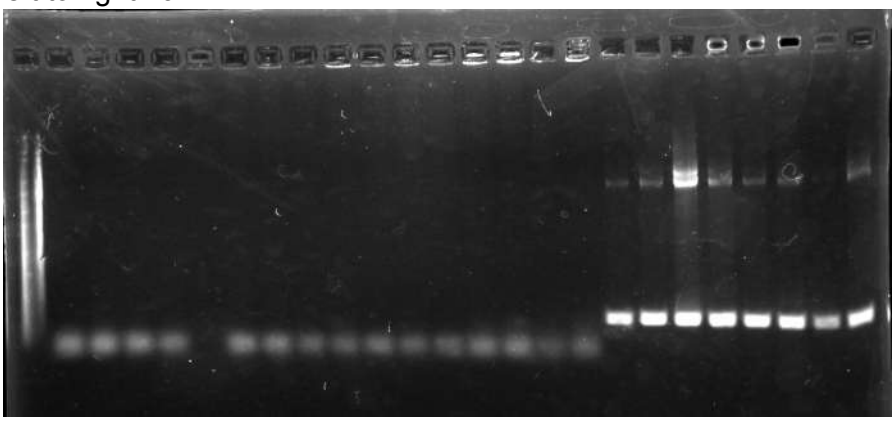
left to right 1-24



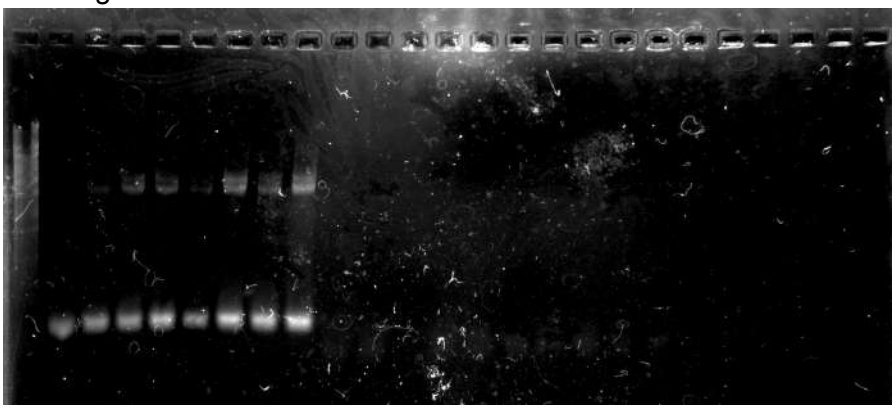
left to right 25-48



left to right 49-72



left to right 73-96



1 & 3 (A) would be a success !!!!!!!

0920

retry gblock PCR (parB_1,parB_2)

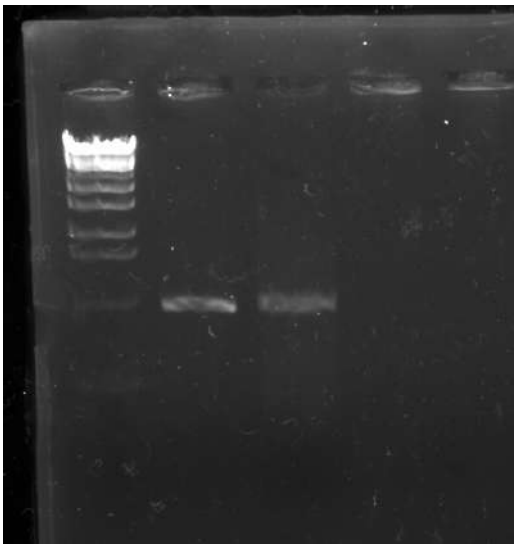
template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size

CFPparB_1	gBlock PCR	f029	58	f030	60.5	60	903
CFPparB_2	gBlock PCR	f031	60.5	f002	60.6	60	921

template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	11.2ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	30sec	20 cycle
extension	68	1min	↓
hold	22	∞	

electrophoresis 50V 40min
1.5% LMP



looks good.

after gel purification, measured the dsDNA concentration

サンプル名	核酸(ng/uL)
CFPparB_1	4.14
CFPparB_2	4.92

miniprep
A1, A3

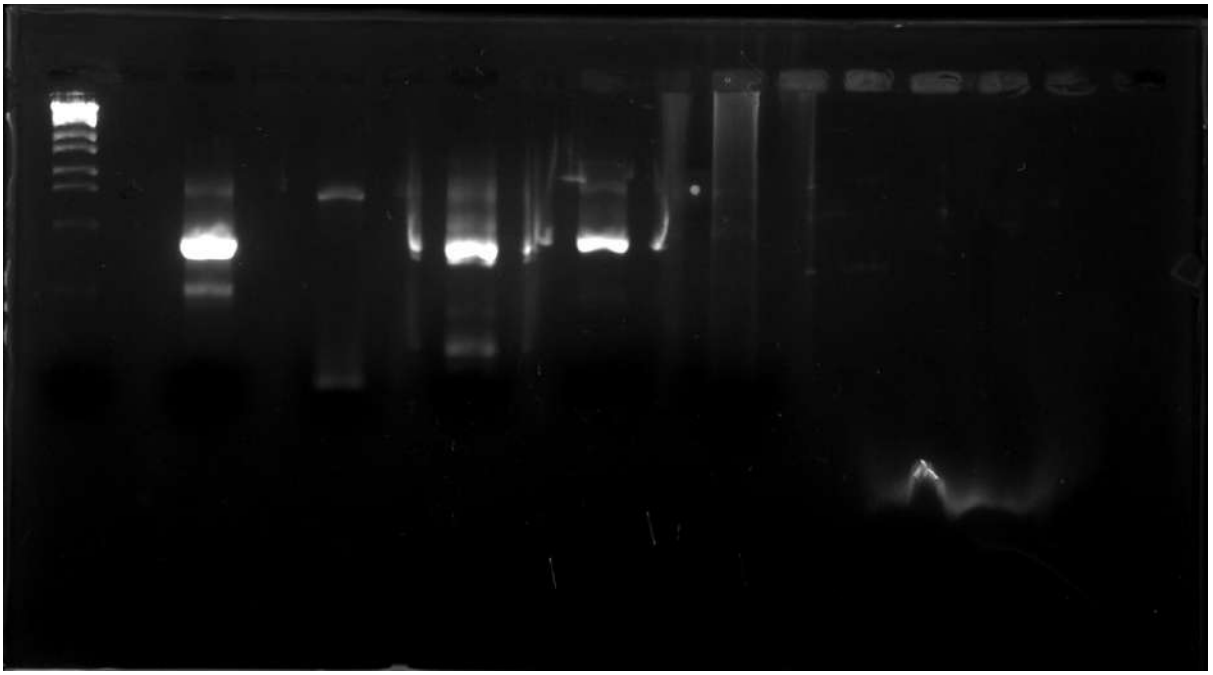
OE-PCR

No.	plasmid	template1	template2	template3	template4	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
21	A	iRFP	oligo_A	sfGFP		f001	60.1	f018	51.8	56	1875
22	B	oligo_B_2	cI-1	DT2	sopC	f060	65.9	f050	55.5	60	1515
23	C	oligo_parS	DT_1	TetR1		f001	60.1	f045	68.5	64	901
24	C	DT_1	TetR1	oligo_C		f026	54.9	f063	65	60	918
25	C	TetR1	oligo_C	CFPparB_1		f027	70.8	f030	60.5	65	1632
26	C	oligo_C	CFPparB_1	CFPparB_2		f062	65	f002	60.6	63	1893
3	B	gB_lp-ecfp-sopB_2	gB_lp-ecfp-sopB_1			f001	60.1	f021	53.3	57	1798
5	B	oligo_B_1	oligo_B_2			f058	62.8	f061	65.9	64	192

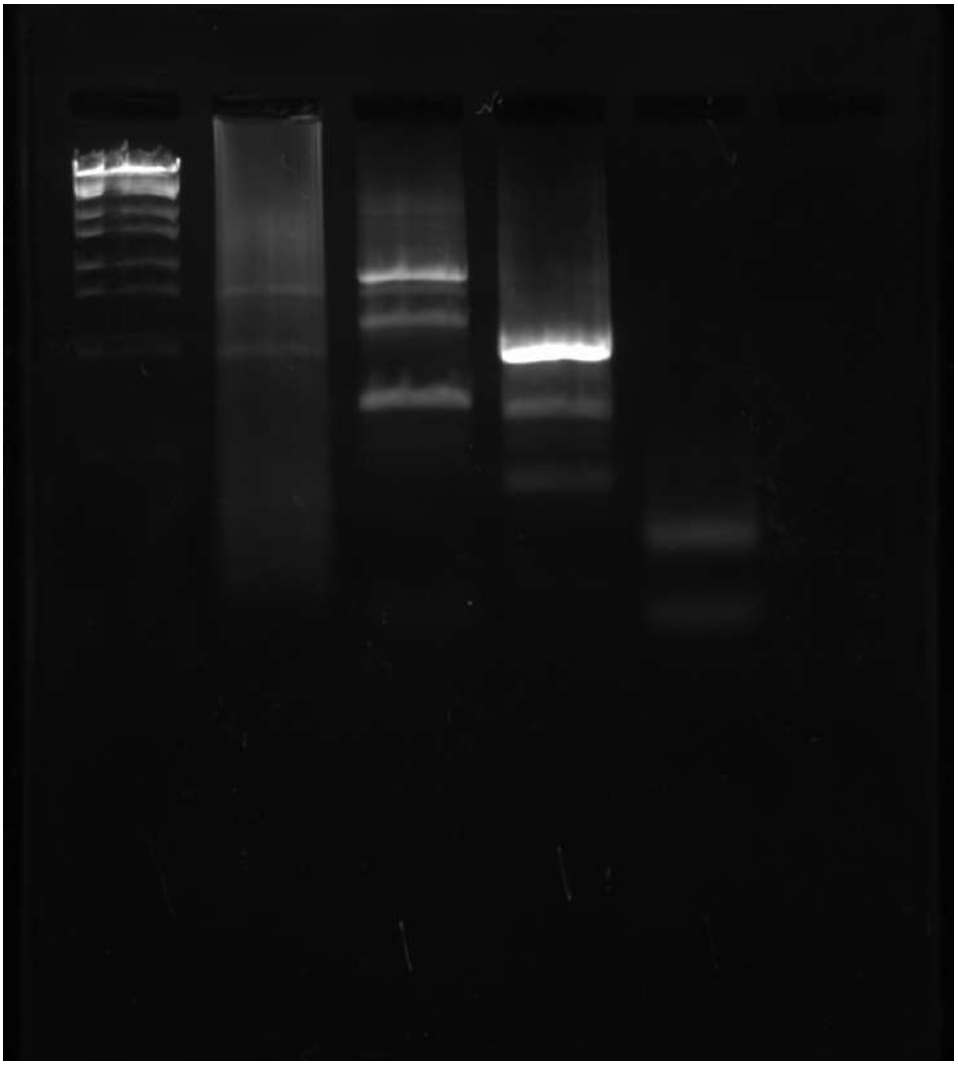
components	conc.	volume (uL)	final conc.
distilled water		12	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

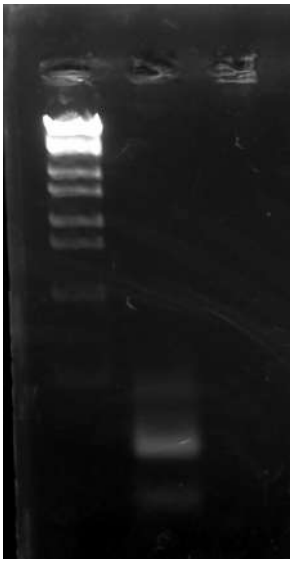
electrophoresis 50V 40min
1.5% LMP
left to right 21-26,3



retry electrophoresis for OE-25, OE-26 ,OE-3, OE-5



electrophoresis 50V 40min
1.5% LMP
sample 5



after gel purification, measured dsDNA concentration.

サンプル名	核酸(ng/uL)
5	5.16
21	63.8
22	3.66
23	37.6
24	26.4

retry OE-PCR

25	C	TetR 1	oligo _C	CFPp arB_1		f027	70.8	f030	60.5	65	1632
26	C	oligo _C	CFPp arB_1	CFPp arB_2		f062	65	f002	60.6	63	1893
3	B	gB_l p-ecf p-sop B_2	gB_lp -ecfp- sopB _1			f001	60.1	f021	53.3	57	1798
5	B	oligo _B_1	oligo _B_2			f058	62.8	f061	65.9	64	192

components	conc.	volume (uL)	final conc.
distilled water		12	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	

total		20	

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

0921

PCR(cl_2, new oligo_D)

cl_2

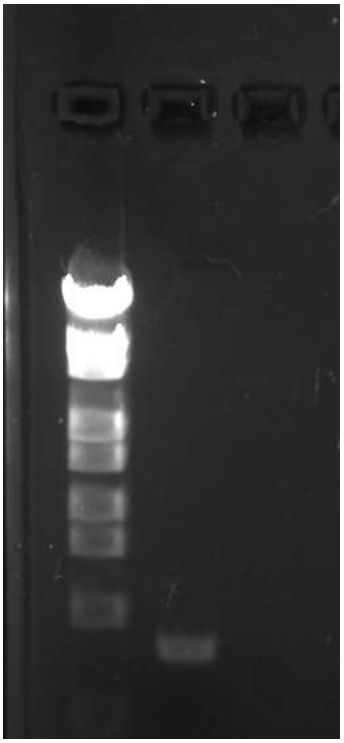
template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

PCR program

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size

CI_2	f107		f023			776



looks good.
after column purification, use as PCR template

サンプル名	核酸(ng/uL)	A260/A280	A260/A230
cI_2	24.9	2.01	2.15

new oligo_D

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

PCR program

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

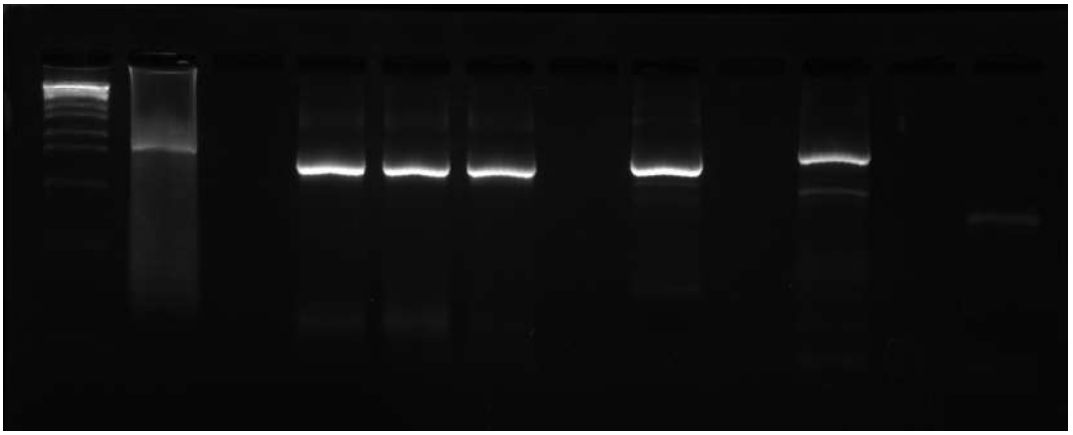
template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
CI_2	f106		f023			827

OE-PCR

No.	plasmid	template1	template2	template3	template4	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
13	C	CFPparB_1	CFPparB_2			f029	58	f002	60.6		1794
22	B	oligo_B_2	cI-1	DT2	sopC	f060	65.9	f050	55.5	60	1515
25	C	TetR1	oligo_C	CFPparB_1		f027	70.8	f030	60.5	65	1632
26	C	oligo_C	CFPparB_1	CFPparB_2		f062	65	f002	60.6	63	1893

electrophoresis 50V 40min
1.5% LMP

left to right 13,22,22,22,25,26,new oligo_D



looks good.
after gel purification, measured dsDNA concentration.

0922

サンプル名	核酸(ng/uL)
13	12.1
22(×1)	too high concentration
22(×1/10)	14.0
25	85.6
26	55.4
new oligo_D	4.90

Infront Assembly

Infront Assembly protocol

0.
set the thermal cycler program as follows;
This helps you immediately conduct step2 after step1.

℃	time
37	hold
37	5min

65	5min
4	hold

1.
Mix the fragments (about 10-30 ng/microL) and 2x IA KKT (Infront Assembly 2x mix) on ice and transfer it to the PCR tube.

1. plasmid B

components	conc.	volume (uL)
gBlock sopB2	10ng/uL	1
OE-PCR 4	10ng/uL	1
OE-PCR 22	10ng/uL	1
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	4

2. plasmid B control1

components	conc.	volume (uL)
DW		3
pSB4K5	20ng/uL	1
2× IA KKT	2x	4

3. plasmid B control2

components	conc.	volume (uL)
gBlock sopB2	10ng/uL	1
OE-PCR 4	10ng/uL	1
DW	10ng/uL	1
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	4

OE-PCR(plasmid D)

components	conc.	volume (uL)	final conc.
distilled water		12	

5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

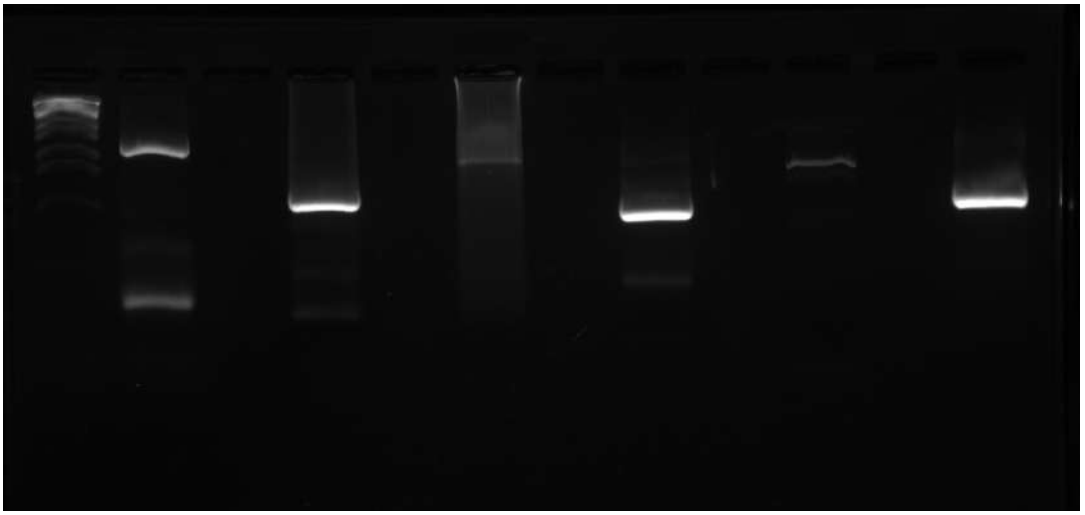
No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
14	D	oligo_ParS	DT_1	f032	77.9	f024	55.1		271
19	D	DT_3	CFPparB_1	f024	55.1	f030	60.5		1062
20	D	CFPparB_1	CFPparB_2	f029	58	f002	60.6		1794
27	D	DT_1	TetR2-oligo_D	f026	54.9	f105			891
28	D	TetR2-oligo_D	oligo_D-cl_2	f027	70.8	f023	70.1		1524
29	D	oligo_D-cl_2	DT_3	f044→f106		f046	62.1		981

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓

hold	16	∞	

0923

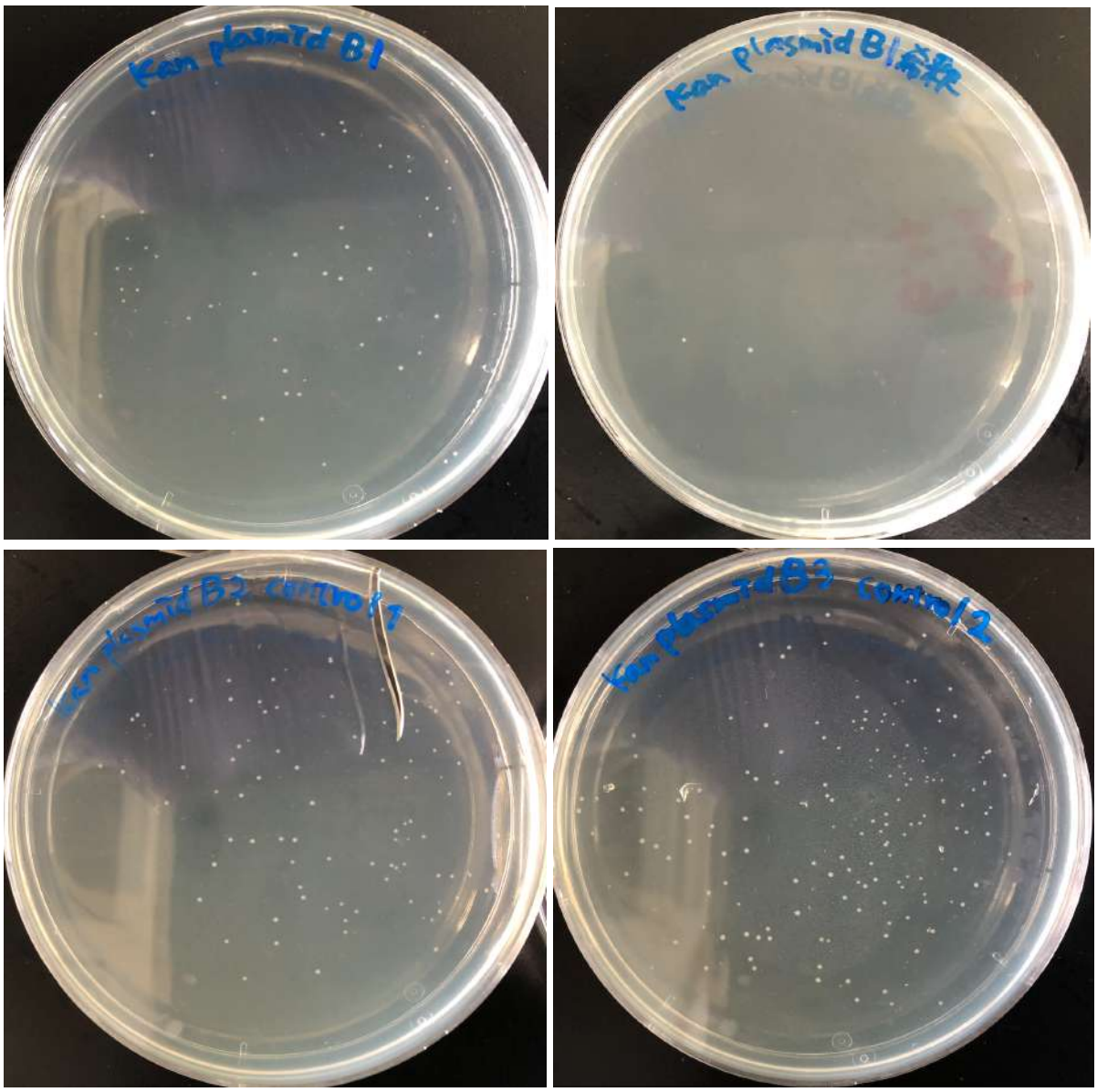
electrophoresis (OE-PCR 14,19,20,27,28,29)
50V 40min 1.5% LMP
left to right OE-PCR 14,19,20,27,28,29

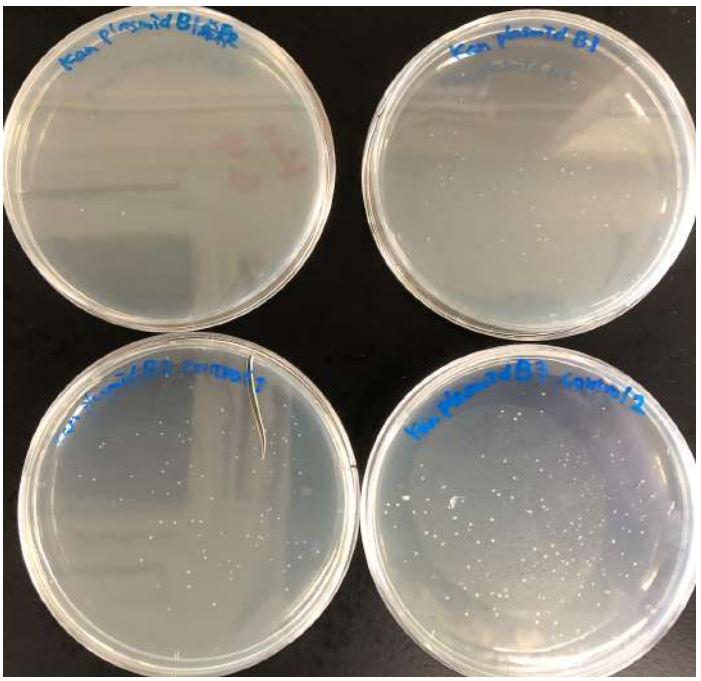


after gel purification, measured dsDNA concentration

サンプル名	核酸(ng/uL)
14	18.2
19	44.8
20	11.6
27	78.4
29	65.4

colony


Kan plasmid B1
Kan plasmid B1
Kan plasmid B2 control 1
Kan plasmid B2 control 2

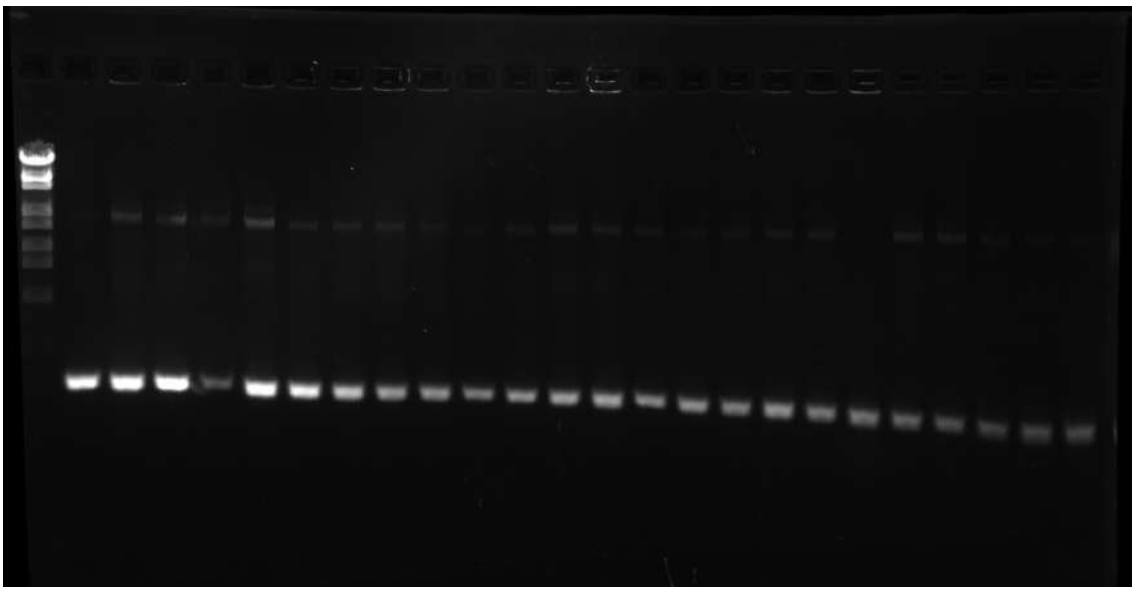


colony PCR

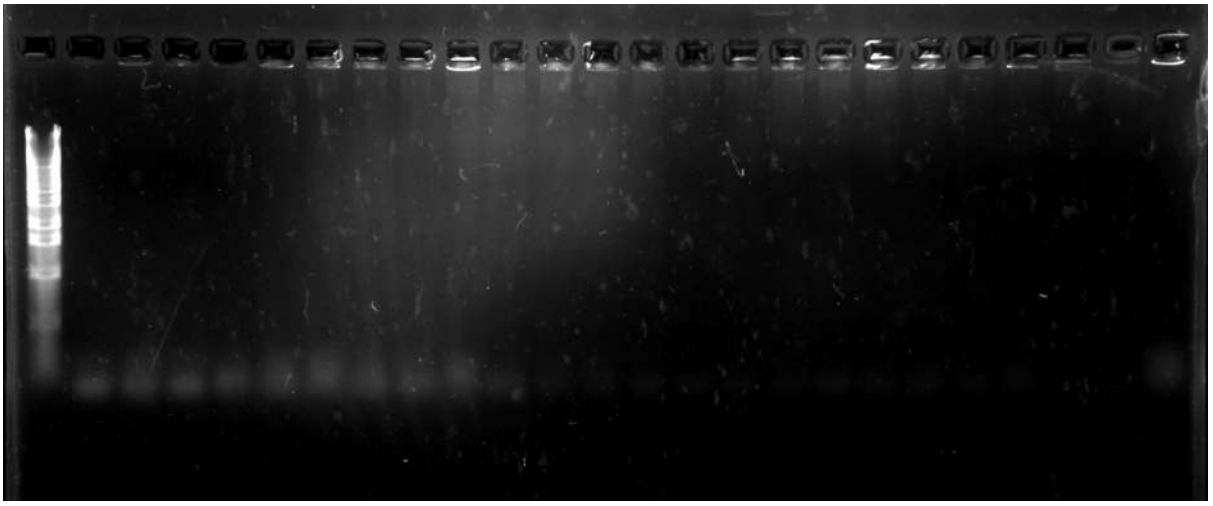
steps	℃	time	
predenature	95	5min	
denature	98	10sec	↑
annealing	55	30sec	35 cycle
extension	72	4min	↓
hold	16	∞	

No.	plasmid	F primer ID	R primer ID	product size
1-16	B	f089	f090	3587
17-20	B control1	f089	f090	
21-24	B control2	f089	f090	
25-40	B	f058	f090	1696
41-44	B control1	f058	f090	
45-48	B control2	f058	f090	

left to right 1-24



left to right 25-48



No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
20	D	CFPpaB_1	CFPpaB_2	f029	58	f002	60.6		1794

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	20 cycle
extension	68	2min	↓

hold	16	∞	

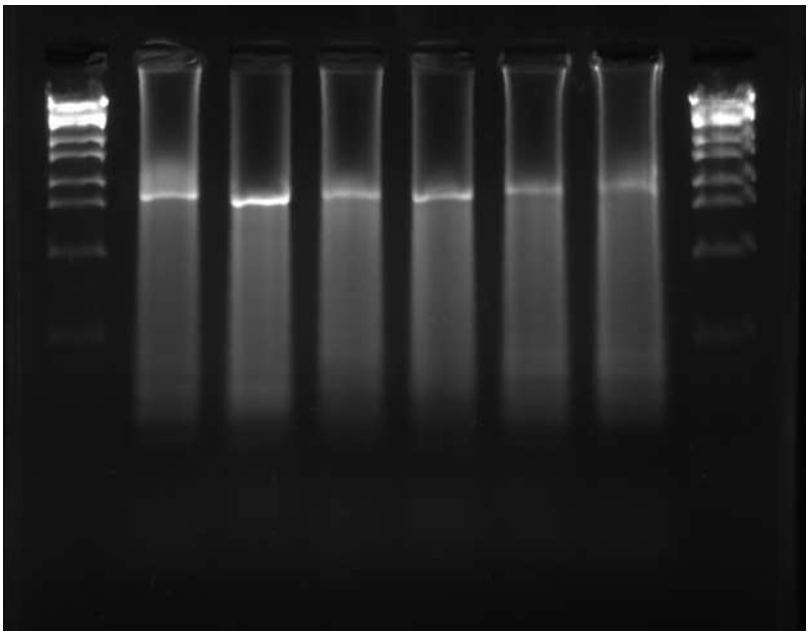
steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	25 cycle
extension	68	2min	↓
hold	16	∞	

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	2min	↓
hold	16	∞	

components	conc.	volume (uL)	final conc.
distilled water		12	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

2 lanes for each sample

from left to right: 25cycle,25,20cycle,20,30cycle,30,



OE-PCR20 gel purification
24.5ng/uL

0924

OE-PCR

No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
30	B	gB sopB 2	gB-sopB1	oligo B-1	f001	60.1	f059	71.7	66	1884

components	conc.	volume (uL)	final conc.
distilled water		12	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM

R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

OE-PCR

No.	plasmid	template1	template2	template3	template4	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
3	B	gB_lp-ecfp-sopB_2	gB_lp-ecfp-sopB_1			f001	60.1	f021	53.3	57	1798

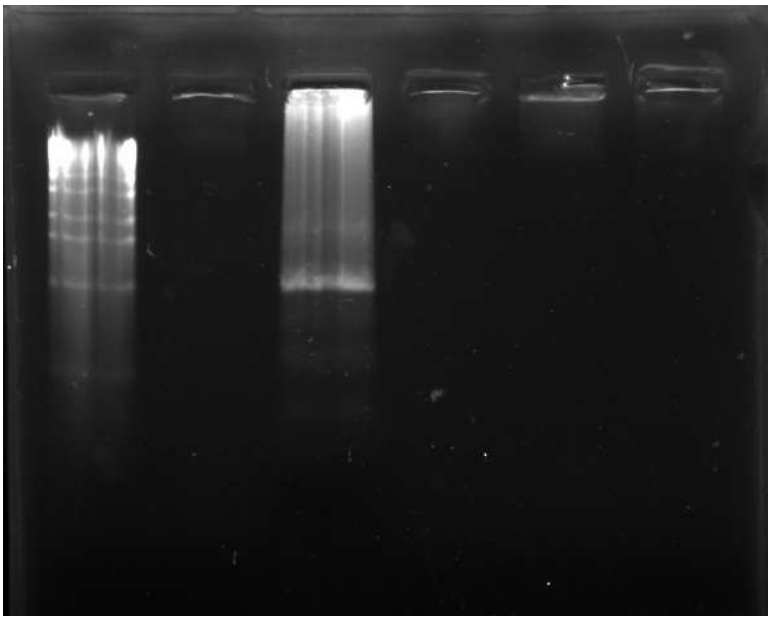
steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	3min	↓
hold	16	∞	

components	conc.	volume (uL)	final conc.
distilled water		12.4	

5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

0925

electrophoresis 50V 40min
1.5% LMP
OE-PCR 3
approximate product size 1798….



ligation

components	volume(uL)
ligation high	2
sma plasmid(100ng/ul)	1
PCR product*	1

1.after ligation reaction, incubate at 16℃ for 1h

2.add 2ul of the reaction mix to 10ul DH5a
3.incubate on ice for 20 min
4.heat-shock at 42℃ for 45sec
5.incubate on ice for 1 min
6.add 100ul of SOC (10x volume of competent cells)
7.incubate at 37℃ for 1h
8.spread it onto LB plate(including proper antibiotics)
9.incubate at 37℃. o/n

No.	plasmid	PCR-product	approximate product size
1	B1-B2	OE-PCR 3	1798
2	C5-C6/ D7-D8	OE-PCR 13/20	1794
3	B4-B7	OE-PCR 22	1515
4	C1-C3	OE-PCR 23	901
5	C4-C6	OE-PCR 26	1893

retry OE-PCR30 Tm60

No.	plasmid	template1	template2	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
30	B	gB sopB 2	gB-sopB1	oligo B-1	f001	60.1	f059	71.7	66	1884

components	conc.	volume (uL)	final conc.
distilled water		12	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	

PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

PCR(sopB_1,sopB_2)

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
CFPsopB_1	gBlock PCR	f020	55.9	f021	53.3	55	977
CFPsopB_2	gBlock PCR	f001	60.1	f019	62	60	851

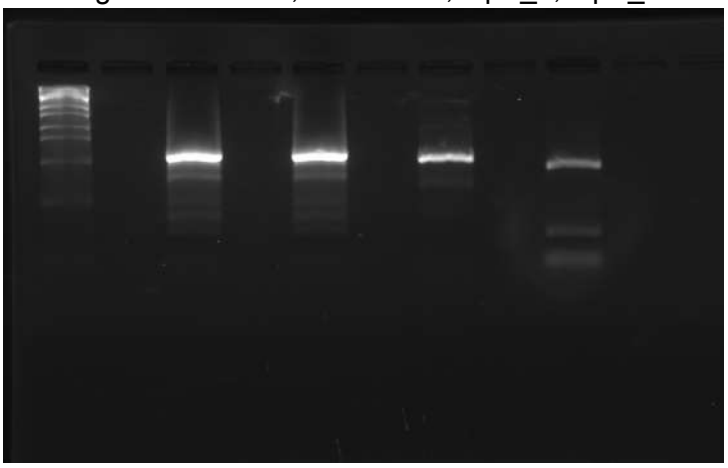
template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	11.2ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑

annealing	60	30sec	16 cycle
extension	68	1min	↓
hold	22	∞	

0926

electrophoresis 50V 40min
1.5% LMP
left to right OE-PCR30,OE-PCR30,sopB_1,sopB_2



sopB_1 & sopB_2 are correct, OE-PCR30 are too short.
after gel purification(sopB_1,sopB_2), measured dsDNA concentration.

サンプル名	核酸(ng/uL)
sopB_1	22.4
sopB_2	9.82

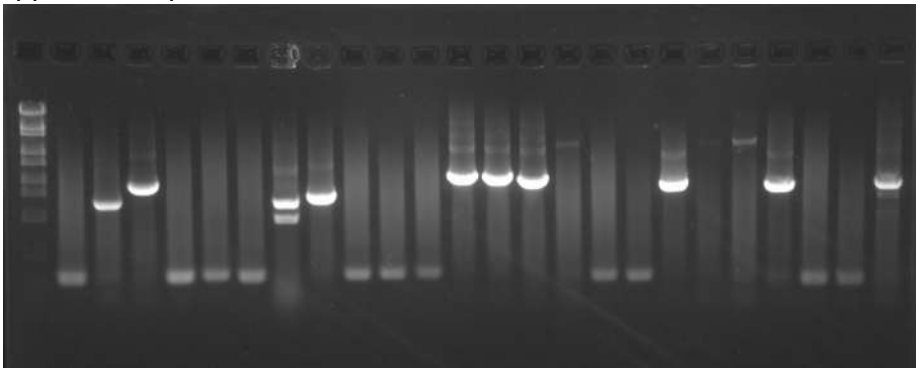
colony PCR

spread 80ul X-gal per one plate

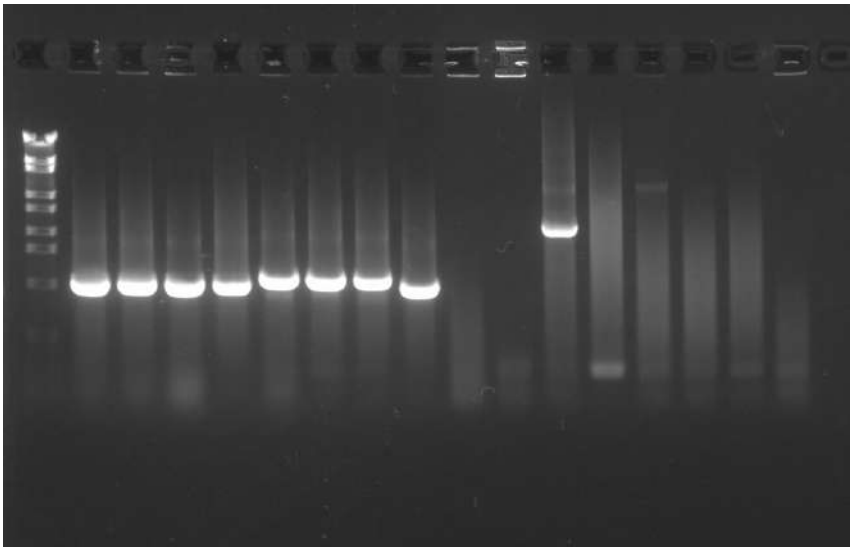
steps	℃	time	
predenature	95	5min	
denature	98	10sec	↑
annealing	55	30sec	35 cycle
extension	72	4min	↓
hold	16	∞	

No.	plasmid	F primer ID	R primer ID	product size
1-8	OE-PCR 3	M13 nF	M13 nRv	1798
9-16	OE-PCR 13/20	M13 nF	M13 nRv	1794
17-24	OE-PCR 22	M13 nF	M13 nRv	1515
25-32	OE-PCR 23	M13 nF	M13 nRv	901
33-40	OE-PCR 26	M13 nF	M13 nRv	1893

left to right 1-24
approximate product size 1-8:1798, 9-16: 1794, 17-24:1515



left to right 25-40
approximate product size 25-32:901, 33-40:1893



miniprep No.1(3,8) No.2(9,10,11) No.3(18,21)No.4(29,31)No.5(35)

0927

OE-PCR(plasmid D three template)

No.	plasmid	template1	template2	template3	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
31	D	oligo_ParS	DT_1	TetR2-oligo_D	f032		f105			973
32	D	DT_1	TetR2-oligo_D	oligo_D-CI_2	f026		f023			1683
33	D	TetR2-oligo_D	oligo_D-CI_2	DT_3	f027		f046			1683
34	D	oligo_D-CI_2	DT_3	ParB_1	f106		f030			1854
35	D	DT_3	ParB_1	ParB_2	f024		f002			1953

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

0928

ligation

ligation

components	volume(uL)

ligation high	2
sma plasmid(100ng/ul)	1
PCR product*	1

after ligation reaction, incubate at 16℃. o/n

No.	plasmid	PCR-product	approximate product size
1	B1-B2	OE-PCR 3	1798
2	B4-B7	OE-PCR 22	1515

→0929 transformation

0929

transformation

2.add 2ul of the reaction mix to 10ul DH5a
3.incubate on ice for 20 min
4.heat-shock at 42℃ for 45sec
5.incubate on ice for 1 min
6.add 100ul of SOC (10x volume of competent cells)
7.incubate at 37℃ for 1h
8.spread it onto LB plate(including proper antibiotics, spread 80ul X-gal)
9.incubate at 37℃. o/n

PCR（new primer:backbone)

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul

Total	20uL

PCR program:

backbone

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	3.5min	↓
hold	16	∞	

template			F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
C	pSB3C5	BioBrick PCR	f004	59.9	f113	47.8		2700
B/D	pSB4K5	BioBrick PCR	f004	59.9	f113	47.8		3381

OE-PCR（new primer）

components	conc.	volume (uL)	final conc.
distilled water		12.4	
5xPrimeSTAR GXL Buffer	5x	4	1x
dNTPs	2.5mM	1.6	200uM each
F primer	10uM	0.4	0.2uM
R primer	10uM	0.4	0.2uM
Template Mixture	*	*	
PrimeSTAR GXL DNA polymerase		0.4	

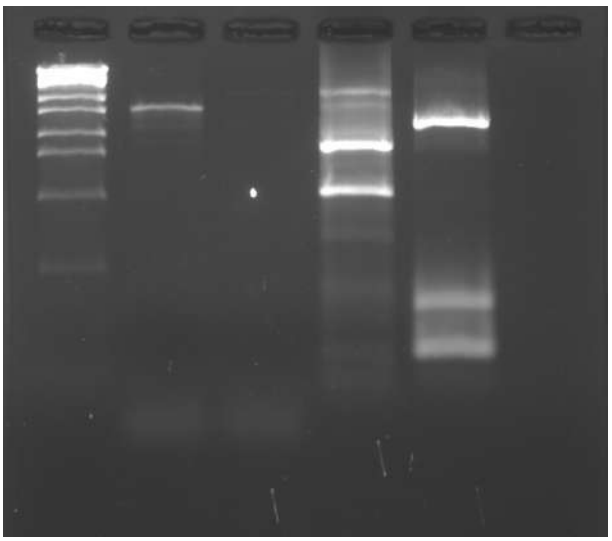
total		20	

PCR program

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	35 cycle
extension	68	2min	↓
hold	16	∞	

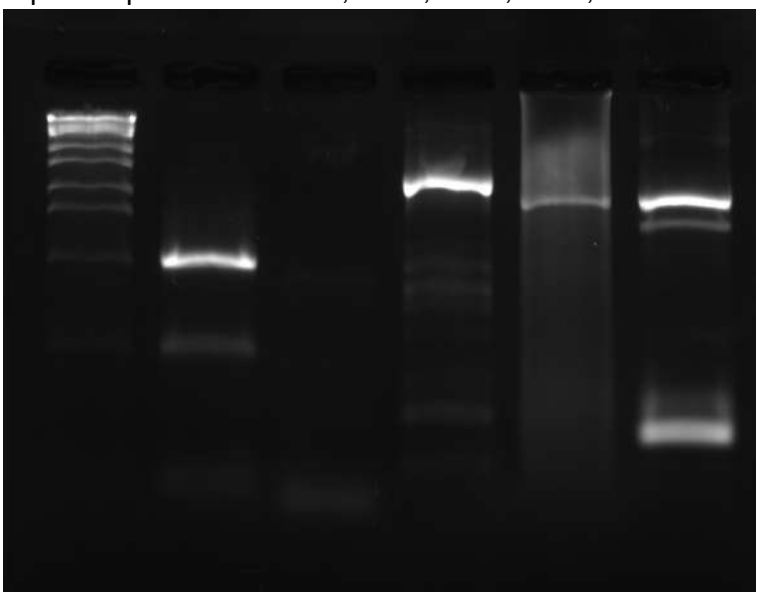
No.	plasm id	templ ate1	templ ate2	F prime r ID	F prime r Tm	R prime r ID	R prime r Tm	Tm condi tion	approximat e produ ct size
103	B	sopB _2	sopB _1	f114	69	f021	53.3		1828
109	C/ D	ParS	DT1	f114	69	f024	55.1		301

electrophoresis 50V 40min
1.5% LMP
from left to right pSB3C5, pSB4K5, OE-103, 109
expected product size 2700, 3381, 1828, 301



The only pSB3C5 band looks good.
after gel purification, measured dsDNA concentration(only pSB3C5)

electrophoresis 50V 40min
1.5% LMP
from left to right OE 31-35
expected product size 973, 1683, 1683, 1854, 1953

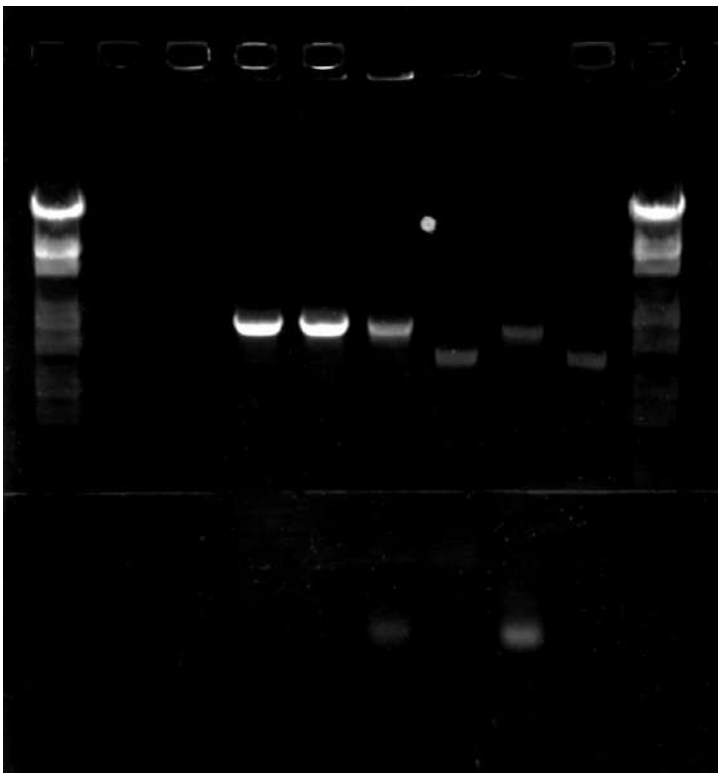


all bands are not correct…

0930

colony PCR

from left to right



all bands are not correct...

retry PCR（new primer:backbone)

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	3.5min	↓
hold	16	∞	

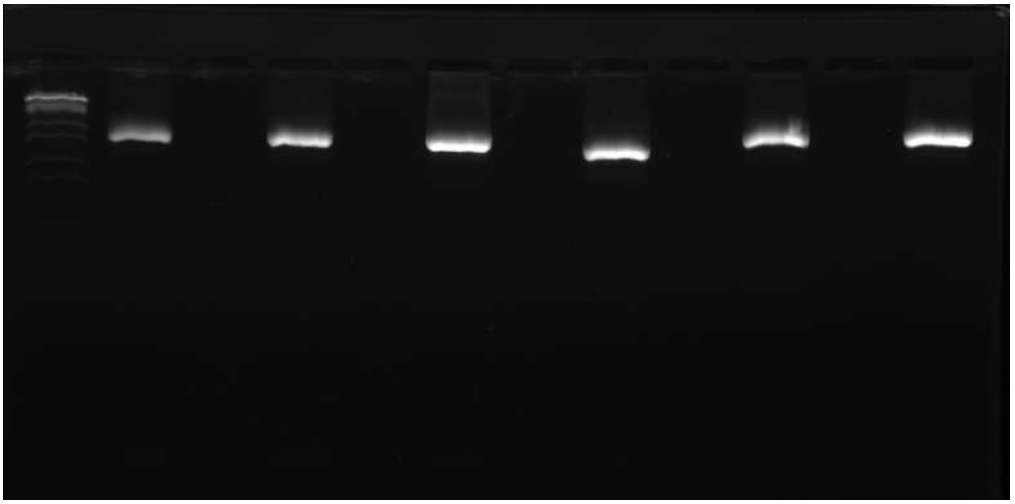
steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	45	15sec	30 cycle
extension	68	3.5min	↓
hold	16	∞	

template(1ng/ul)	0.4ul

primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

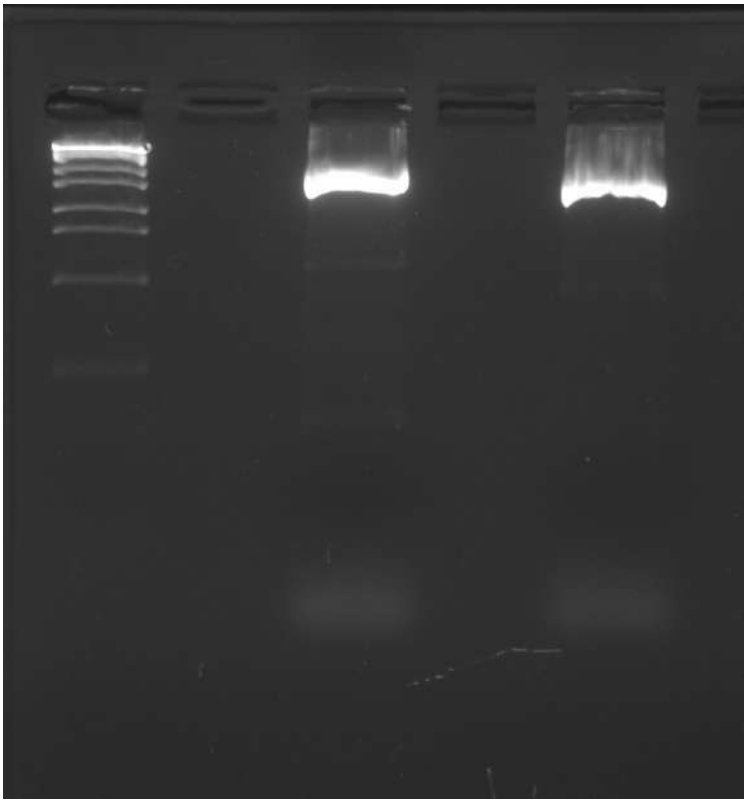
template		F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
B/D	pSB4K5(circle)	BioBrick PCR	f004	59.9	f113	47.8	3381
B/D	pSB4K5(digested with Nco1)	BioBrick PCR	f004	59.9	f113	47.8	3381
B/D	pSB4K5(digested with HincII)	BioBrick PCR	f004	59.9	f113	47.8	3381
C	pSB3C5(digested with Nco1)	BioBrick PCR	f004	59.9	f113	47.8	2700

→electrophoresis 50V 50min
from left to right Tm=45:pSB4K5(circle, digested with Nco1,digested with HincII), pSB3C5, Tm=55:pSB4K5(circle, digested with Nco1)



looks good!!

from left to right Tm=55:pSB4K5(digested with HincII), pSB3C5



looks good!!

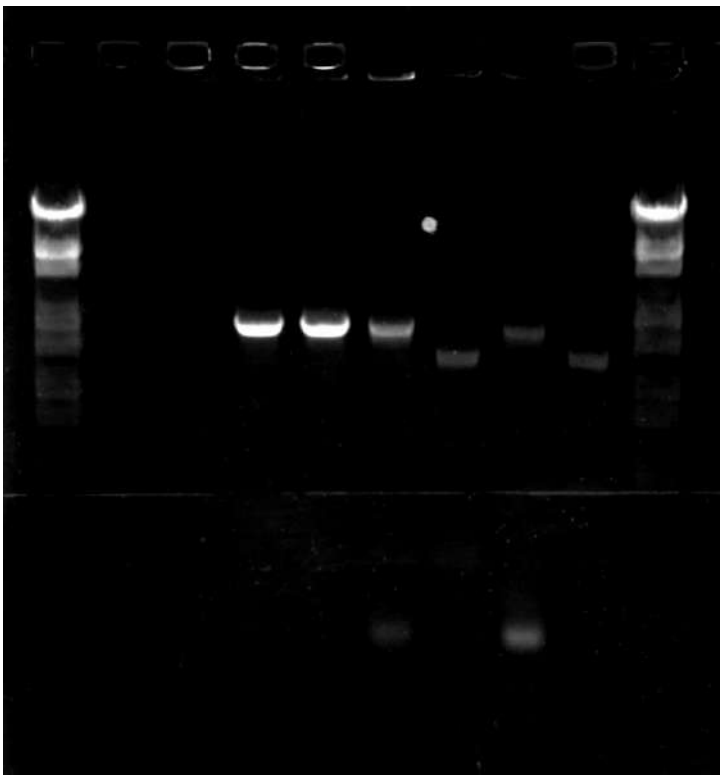
colony PCR

spread 80ul X-gal per one plate

steps	℃	time	
predenature	95	5min	
denature	98	10sec	↑
annealing	55	30sec	35 cycle
extension	72	4min	↓
hold	16	∞	

No.	plasmid	F primer ID	R primer ID	product size
	OE-PCR 3	M13 nF	M13 nRv	
	OE-PCR 22	M13 nF	M13 nRv	

from left to right



1001

retry blunt-end cloning

puC118(1040ng/ul)	1ul
bufferT	2ul
Sma1	0.5ul
BSA(0.1%)	2ul
DW	14.5ul
total	20ul

colony PCR

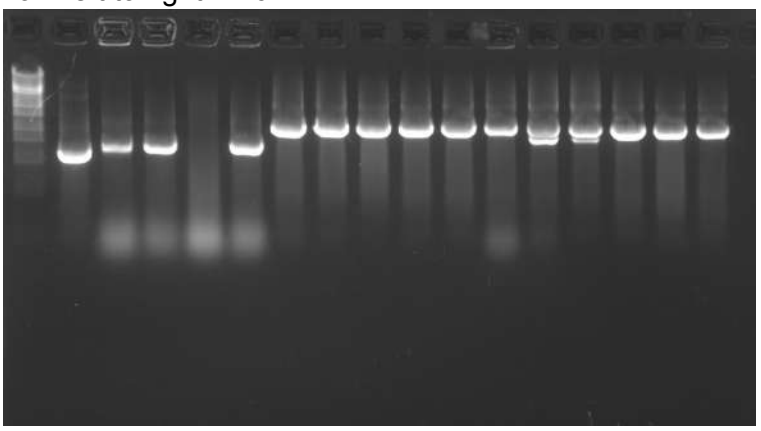
used colonies that fluorenced by UV

steps	℃	time	
predenature	95	5min	
denature	98	10sec	↑

annealing	55	30sec	35 cycle
extension	72	4min	↓
hold	16	∞	

No.	plasmid	F primer ID	R primer ID	product size
1-5	OE-PCR 3	M13 nF	M13 nRv	
6-16	OE-PCR 13/20	M13 nF	M13 nRv	

electrophoresis 100V 20 min
from left to right 1-16



→ No.6, 16 : cultured liquid LB (including ampicillin)

1002

gBlock PCR (new primer:insert)

template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul

DW	11.2ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	30sec	16 cycle
extension	68	1min	↓
hold	22	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
B	CFPsopB_2	gBlock PCR	f114	69	f019	62		881

PCR (new primer:oligo_ParS)

PCR program:

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑

annealing	60	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
oligo_ParS(gel purified)	f114	69	f033	75		142

1005

PCR

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	1min50sec	↓
hold	16	∞	

No.	plasmid	template1	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
20	C/D	OE-2011-16	f029	58	f002	60.6	55	1794

1006

electrophoresis
1.5% LMP
OE-20 11-16
expected production size 1794

retry-PCR

gBlock PCR (new primer:insert)

template(1ng/ul)	2ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4u
DW	11.2ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	30sec	16 cycle
extension	68	1min	↓
hold	22	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
B	CFPsopB_2	gBlock PCR	f114	69	f019	62		881

PCR (new primer:oligo_ParS)

PCR program:

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul

dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
oligo_ParS(gel purified)	f114	69	f033	75		142

PCR

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	1min50sec	↓
hold	16	∞	

No.	plasmid	template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
20	C/D	OE-2011-16	f029	58	f002	60.6	55	1794

gel purification

サンプル名	核酸(ng/uL)
pSB4K5(circle) Tm=45	18.3
pSB4K5(digested with Nco1)Tm=45	25.6
pSB4K5(digested with HincII)Tm=45	65.4
pSB3C5(digested with Nco1)Tm=45	28.6
pSB4K5(circle) Tm=55	35.2
pSB4K5(digested with Nco1)Tm=55	20.4
pSB4K5(digested with HincII)Tm=55	95.0
pSB3C5(digested with Nco1)Tm=55	45.6

Infront Assembly

Infront Assembly protocol

0.
set the thermal cycler program as follows;
This helps you immediately conduct step2 after step1.

℃	time
37	hold
37	5min
65	5min
4	hold

1.
Mix the fragments (about 10-30 ng/microL) and 2x IA KKT (Infront Assembly 2x mix) on ice and transfer it to the PCR tube.

1. **plasmid B**

components	conc.	volume (uL)
new sopB2	10ng/uL	1
OE-PCR 4	10ng/uL	1
OE-PCR 6	10ng/uL	1
OE-PCR 8	10ng/uL	1
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	5

2. **plasmid B control1**

components	conc.	volume (uL)
DW		4
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	5

3. **plasmid B control2**

components	conc.	volume (uL)
new sopB2	10ng/uL	1
OE-PCR 4	10ng/uL	1
OE-PCR 6	10ng/uL	1
DW	10ng/uL	1
linearized pSB4K5	20ng/uL	1
2× IA KKT	2x	5

linearized pSB4K5 was digested with NcoI and amplified by PCR (Tm=55)

4. **plasmid C**

components	conc.	volume (uL)
new oligo_ParS	10ng/uL	1
OE-PCR 24	10ng/uL	1
OE-PCR 20 11-16	10ng/uL	1
linearized pSB3C5*	20ng/uL	1
2× IA KKT	2x	4

5. **plasmid C control 1**

components	conc.	volume (uL)

DW		3
linearized pSB3C5*	20ng/uL	1
2× IA KKT	2x	4

6. plasmid C control 2

components	conc.	volume (uL)
OE-PCR 24	10ng/uL	1
OE-PCR 20 11-16	10ng/uL	1
DW	10ng/uL	1
linearized pSB3C5*	20ng/uL	1
2× IA KKT	2x	4

linearized pSB3C5 was digested with NcoI and amplified by PCR (Tm=55)

7. plasmid D

components	conc.	volume (uL)
new oligo_ParS	10ng/uL	1
OE-PCR 27	10ng/uL	1
OE-PCR 29	10ng/uL	1
OE-PCR 20 11-16	10ng/uL	1
linearized pSB4K5*	20ng/uL	1
2× IA KKT	2x	5

8. plasmid D control 1

components	conc.	volume (uL)
DW		4
linearized pSB4K5*	20ng/uL	1
2× IA KKT	2x	5

9. plasmid D control 2

components	conc.	volume (uL)
OE-PCR 27	10ng/uL	1
OE-PCR 29	10ng/uL	1
OE-PCR 20 11-16	10ng/uL	1
DW		1

linearized pSB4K5*	20ng/uL	1
2× IA KKT	2x	5

linearized pSB4K5 was digested with NcoI and amplified by PCR (Tm=55)
only plasmid D control 2, use linearized pSB4K5 which digested with NcoI and amplified by PCR (Tm=45)

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infront assembly(plasmid D)

0.
set the thermal cycler program as follows;
This helps you immediately conduct step2 after step1.

℃	time
37	hold
37	5min
65	5min
4	hold

1.
Mix the fragments (about 10-30 ng/microL) and 2x IA KKT (Infront Assembly 2x mix) on ice and transfer it to the PCR tube.

1. **plasmid D**

components	conc.	volume (uL)
new oligo_ParS	10ng/uL	1
OE-PCR 27	10ng/uL	1
OE-PCR 29	10ng/uL	1
OE-PCR 20 11-16	10ng/uL	1
linearized pSB3C5*	20ng/uL	1
2× IA KKT	2x	5

2. **plasmid D control 1**

components	conc.	volume (uL)
DW		4
linearized pSB3C5*	20ng/uL	1
2× IA KKT	2x	5

3. **plasmid D control 2**

components	conc.	volume (uL)
OE-PCR 27	10ng/uL	1
OE-PCR 29	10ng/uL	1
OE-PCR 20 11-16	10ng/uL	1
DW		1
linearized pSB3C5*	20ng/uL	1
2× IA KKT	2x	5

PCR(backbone)

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

template			F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
D	pSB3C5	BioBrick PCR	f004	59.9	f113	47.8		2700

steps	℃	time	
predenature			

denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	3.5min	↓
hold	16	∞	

PCR（OE-20 11-16）

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	55	15sec	30 cycle
extension	68	1min50sec	↓
hold	16	∞	

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

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colony PCR

used colonies that fluorenced by UV

steps	℃	time	

predenature	95	5min	
denature	98	10sec	↑
annealing	55	30sec	35 cycle
extension	72	4min	↓
hold	16	∞	

No.	plasmid	F primer ID	R primer ID	product size
1-22	D	f089	f090	3909
23-24	D control 2	f089	f090	
25-46	D	f089	f023	1872
47-48	D control 2	f089	f023	

electrophoresis 100V 20 min

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PCR (except for Double Terminator)

template(1ng/ul)	0.4ul
primestar GXL DNA pol	0.4ul
dNTP Mixture(2.5uM each)	1.6ul
5xPS GXL buffer	4ul
primer1 (10uM)	0.4ul
primer2 (10uM)	0.4ul
DW	12.8 ul
Total	20uL

steps	℃	time	
predenature			
denature	98	10sec	↑
annealing	60	15sec	30 cycle
extension	68	1min	↓
hold	16	∞	

name	plasmid	template1	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	approximate product size
ParS_eDT long	D	ParS	f032	80.8	f125	71.8		149
ParS_eDT short	D	ParS	f032	80.8	f127	66.6		137
oligoD-CI_eDT long	D	new oligoD-CI	f106	74.6	f126	68.9		849
oligoD-ParS_eDT short	D	new oligoD-CI	f106	74.6	f128	60.0		847