Lab note iGEM Kyoto 2021
Yuto Ueda

08132021

#Production platform

・Agarose gel electrophoresis

to verify oligo no-insert PCR & gBlock PCR products by Liu (refer to “20210813, oligo no-insert PCR & gBlock PCR” Alex Liu).

samples:

sample	amplicon size	sample	amplicon size
oligo_A in plasmid A	137	lp-ecfp-parB_2 in plasmid C	921
oligo_parS in plasmid C	112	lp-ecfp-sopB_1 in plasmid B	977
iRFP in plasmid A	1009	lp-ecfp-sopB_2 in plasmid B	851
lp-ecfp-parB_1 in plasmid C	903		

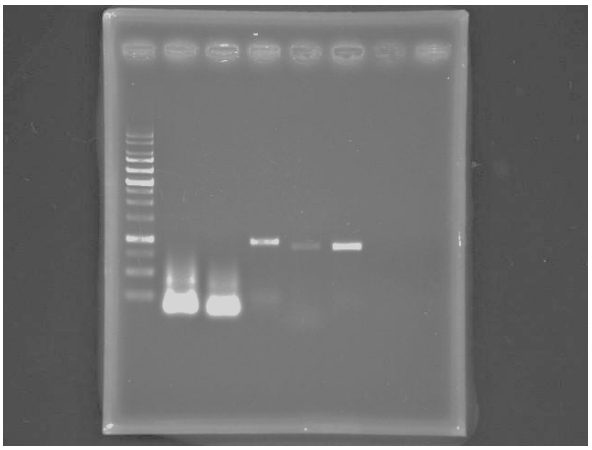
refer to here and plasmid construct in benchling for amplicon size.

parameters:

gel %	1%
run time	25min
Voltage	100V

EtBr-stained for 20min.

Result:



lane1: ladder

lane2: oligo_A
lane3: oligo_parS
lane4: iRFP
lane5: lp-ecfp-parB_2
lane6: lp-ecfp-parB_1
lane7: lp-ecfp-sopB_1
lane8: lp-ecfp-sopB_2

The bands of lane 2 and 3 are smeary because ssDNA oligo no-insert PCR products may work as primer, but the target size band is stronger than the others in lane. Strangely, the products of sopB_1 and sopB_2 are not amplified as the band appears. However, we can use gBlock itself when cloning.

(Amplicons from oligo no-template PCR were not column-purified as they can be diluted and used at low concentration in InFront Assembly reaction due to small size.

just noted that each primer set sopB_1 and sopB_2 were mistakenly used, and that's why PCR failed…Try to be more careful.

Anyway, amplicons from gBlock PCR are not planned to be used in InFront Assembly, but gBlocks themselves are.
Liu 08142021)

08192021

#Production platform

・Miniprep and Measure concentration of pSB1A3-mRFP

Instead of Liu, miniprep the samples of pSB1A3 after "pSB1A3-mRFP (2021 kit plate 6 - 2G) transformation" on 08162021 by Liu. Pick two colonies to miniprep, so the names of samples are pSB1A3-mRFP-1, -2.

pSB1A3-mRFP-1	59.5	ng/ul
pSB1A3-mRFP-2	56.7	ng/ul

08202021

#Production platform

・BioBrick PCR

pSB1A3-mRFP:

template	F primer ID	F primer Tm	R primer ID	R primer Tm	Tm condition	product size
pSB1A3 -mRFP	f004	59.9	f003	60.1	60	2155

miniprepped plasmids were used as PCR template for these.
pSB1A3-mRFP-1 59.5 ng/μl

first, digested with HincII to linearize.

Fast Digest HincII	1uL
10X FD Buffer	2uL
MilliQ	16uL
pSB1A3-mRFP-1 @59.5ng/uL	~~1uL~~ 2ul*
total	~~20uL~~ 21ul

*add 2ul pSB1A3-mRFP-1 into reaction by mistake.
incubated at 37℃ for 30min, then at 65℃ for 5min to heat-inactivate. Between 37℃ and 65℃, store at 4℃ for 4h. And, after heat-inactivating, store at 4℃ for 2h.
After the digestion, the sample was directly used as a PCR template.

PCR program:

steps	℃	time	
predenature	98	1min	
denature	98	10sec	↑
annealing	60	15sec	**30** cycle
extension	68	5min	↓
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
BioBrick	*	0.8	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

*

template BioBrick		quality
HincII-cut pSB1A3	**5ng/uL, 0.5ng/uL, 0.05ng/uL, 0.005ng/uL**	miniprep → digestion
Negative control		

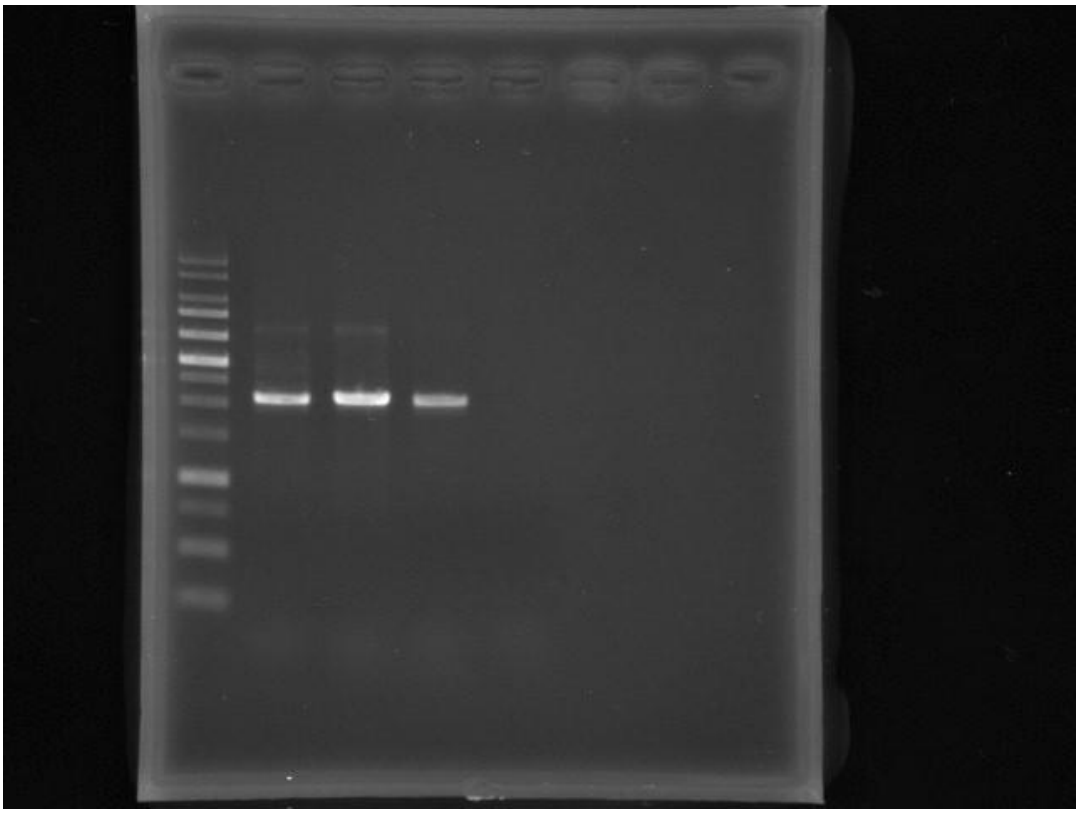
08212021

#Production platform

・BioBrick PCR (following 08202021)

run on 1% agarose gel for 25min at 100V, then EtBr-stained for 30min.

marker: 5ul samples: PCR product 3ul+ loading buffer 1ul



lane	sample	expected size (bp)
1	1kbp marker	10-8-6-5-4-(3)-2.5-2-1.5-(1)-0.75-0.5-0.25 kb
2	HincII-cut pSB1A3-mRFP-1 5ng/ul	2155
3	HincII-cut pSB1A3-mRFP-1 0.5ng/uL	2155
4	HincII-cut pSB1A3-mRFP-1 0.05ng/uL	2155
5	HincII-cut pSB1A3-mRFP-1 0.005ng/ul	2155

looks OK.

because of a less undesired product, choose 0.05ng/ul one of pSB1A3-mRFP-1.

・Dpn1 treatment

treat with an appropriate amount of DpnI for 1h at 37℃.

PCR sample	Dpn1 amount
pSB3C5 (0.05ng/ul)	0.5ul
TetR_082	0.5ul
pSB1A3 (0.05ng/ul)	0.5ul

08222021

#Production platform

・Column Purification

samples treated with Dpn1 by Promega Wizard® SV Gel and PCR Clean-Up System.

9 samples	sfGFP, pSB3C5 (0.05ng/ul), pSB4K5 (0.5ng/ul), TetR, cl_1, cl_2, TetR_082, pSB1A3 (0.05ng/ul), sopC

After column purification, measure the concentration of each sample by Nanodrop 2000.

sample	concentration	apparent amount
sfGFP	190.2 ng/ul	24 ul
pSB3C5 (0.05ng/ul)	69.6 ng/ul	24 ul
pSB4K5 (0.5ng/ul)	41.0 ng/ul	24 ul
TetR	188.3 ng/ul	24 ul
cl_1	139.0 ng/ul	24 ul
cl_2	145.8 ng/ul	24 ul
TetR_082	194.3 ng/ul	24 ul
pSB1A3 (0.05ng/ul)	46.7 ng/ul	24 ul
sopC	114.5 ng/ul	24 ul

08232021

#Production platform

･OE-PCR 001

First, measure the concentration of each sample to add equal moller every PCR amplification sample, and to gain fragments for purpose. The samples consisting of fragments are separated to make 4 groups, which are [Plasmid A], [Plasmid B], [Plasmid C] and [Plasmid D]. So, make a template master mix consisting of each group.

[Plasmid A]

number	sample	concentration	amplicon size
A1	iRFP	6 ng/ul	1009
A2	oligo_A*	8 ng/ul	137
A3	sfGFP	190.2 ng/ul	789

[Plasmid B]

number	sample	concentration	amplicon size
B1	gB_lp-ecfp-sopB_2	10 ng/ul	851
B2	gB_lp-ecfp-sopB_1	10 ng/ul	977
B3	oligo_B_1*	12 ng/ul	116
B4	oligo_B_2*	12 ng/ul	116
B5	cl_1	139.0 ng/ul	750
B6	DT_2*	8 ng/ul	159
B7	sopC	114.5 ng/ul	571

[Plasmid C]

number	sample	concentration	amplicon size
E1	oligo_parS*	8 ng/ul	112
E2	DT_1*	8 ng/ul	189
C1	TetR	188.3 ng/ul	660
C2	oligo_C*	12 ng/ul	129

E3	gB_lp-ecfp-parB_1	10 ng/ul	903
E4	gB_lp-ecfp-parB_2	10 ng/ul	921

[Plasmid D]

number	sample	concentration	amplicon size
E1	oligo_parS*	8 ng/ul	112
E2	DT_1*	8 ng/ul	189
D1	TetR_082	194.3 ng/ul	690
D2	oligo_D*	12 ng/ul	114
D3	cl_2	145.8 ng/ul	780
D4	DT_3*	8 ng/ul	189
E3	gB_lp-ecfp-parB_1	10 ng/ul	903
E4	gB_lp-ecfp-parB_2	10 ng/ul	921

*The concentration of some PCR amplification samples is estimated by comparing each sample with 5ul of 1kb DNA Ladder “BRG-1 kb-02” (Bioregenerations).

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

steps	℃	time	

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

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 MasterMix	*	0.4	
PrimeSTAR GXL DNA polymerase		0.2	
total		**10**	

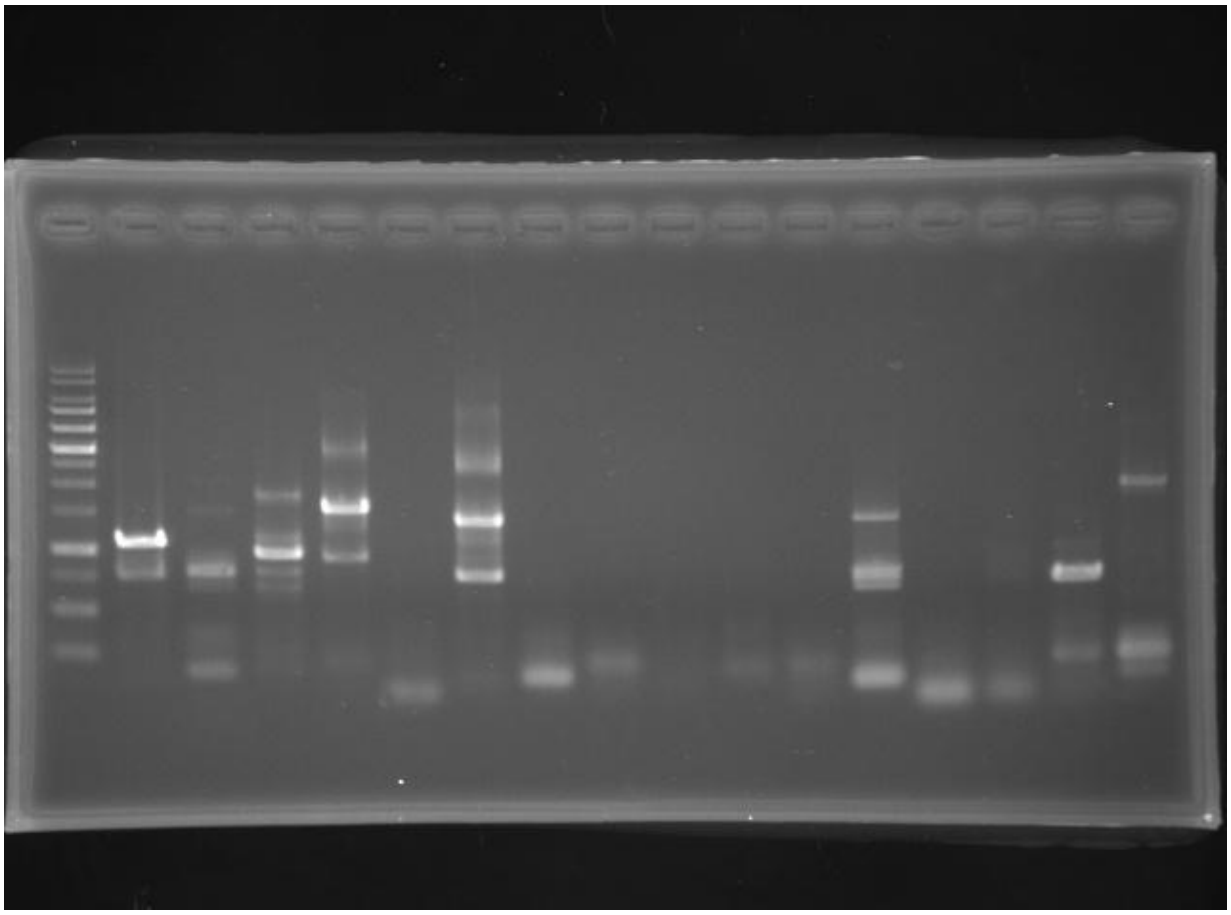
*

Template MasterMix	samples
mA_ori	**A1**(iRFP), **A2**(oligo_A), **A3**(sfGFP)
mB_ori	**B1**(gB_lp-ecfp-sopB_2), **B2**(gB_lp-ecfp-sopB_1), **B3**(oligo_B_1), **B4**(oligo_B_2), **B5**(cl_1), **B6**(DT_2), **B7**(sopC)
mC_ori	**E1**(oligo_parS), **E2**(DT_1), **C1**(TetR), **C2**(oligo_C), **E3**(gB_lp-ecfp-parB_1), **E4**(gB_lp-ecfp-parB_2)
mD_ori	**E1**(oligo_parS), **E2**(DT_1), **D1**(TetR_082), **D2**(oligo_D), **D3**(cl_2), **D4**(

	DT_3), **E3**(gB_lp-ecfp-parB_1), **E4**(gB_lp-ecfp-parB_2)

run on 1% agarose gel for 25min at 100V, then EtBr-stained for 30min.
marker: 5ul samples: PCR product 3ul+ loading buffer 1ul

(08232021 by Alex Liu)
the gel result



samples:

lame	sample	size	lane	sample	size
1	1kbp marker Marker	10-8-6-5-4-(3)-2.5-2-1.5-(1)-0.75-0.5-0.25 kb			
2	A1-A3 in plasmid A	1875	3	B1-B7 in plasmid B	3350
4	B1-B2 in plasmid B	1798	5	B3-B7 in plasmid B	1582
6	B1-B4 in plasmid B	1960	7	B5-B7 in plasmid B	1420
8	E1-E4 in plasmid C	2764	9	E1-C2 in plasmid C	1000
10	E3-E4 in plasmid C	1794	11	E1-C1 in plasmid C	901
12	C2-E4 in plasmid C	1893	13	E1-E4 in plasmid D	3688

14	E1-D2 in plasmid D	1015	15	D3-E4 in plasmid D	2703
16	E1-D3 in plasmid D	1765	17	D4-E4 in plasmid D	1953

The above sample name is described with a primer set, and the amplicon size is referred to here and plasmid constructed in benchling for amplicon size.
The red text sample might look OK.

08242021

#Production platform

･OE-PCR 002

The previous OE-PCR would explore the combination of primer-set to do InFront Assembly of 2 inserts. Because of the previous result, the combination for plasmid A and B are estimated, but fragments for plasmid C cannot be discovered in gel electrophoresis and the band of the other primer-set for plasmid D is not.
Add equal moller every PCR amplification sample, and gain fragments for purpose.

PCR program: **1116b ≦ expected amplicon size ≦ 2523b**

steps	℃	time	
predenature	98	1min	
denature	98	10sec	↑
annealing	55	15sec	**30** cycle
extension	68	3min	↓
hold	16	∞	

PCR program: **expected amplicon size = 271b**

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

reaction composition: **A1-A2, B1-B2, B3-B7**

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 MasterMix	*	0.8	
PrimeSTAR GXL DNA polymerase		0.4	
total		**20**	

reaction composition: **E1-E2, C1-E4, E1-D3, D4-E4**

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 MasterMix	*	0.4	
PrimeSTAR GXL DNA polymerase		0.2	
total		**10**	

*

Template MasterMix	samples
A1-A2	**A1**(iRFP), **A2**(oligo_A)
B1_B2	**B1**(gB_lp-ecfp-sopB_2), **B2**(gB_lp-ecfp-sopB_1)
B3-B7	**B3**(oligo_B_1), **B4**(oligo_B_2), **B5**(cl_1), **B6**(DT_2), **B7**(sopC)
E1-E2	**E1**(oligo_parS), **E2**(DT_1)
C1-E4	**C1**(TetR), **C2**(oligo_C), **E3**(gB_lp-ecfp-parB_1), **E4**(gB_lp-ecfp-parB_2)
E1-D3	**E1**(oligo_parS), **E2**(DT_1), **D1**(TetR_082), **D2**(oligo_D), **D3**(cl_2)
D4-E4	**D4**(DT_3), **E3**(gB_lp-ecfp-parB_1), **E4**(gB_lp-ecfp-parB_2)

run on 1% agarose gel for 25min at 100V, then SYBR-Safe-stained for 30min.

marker: 5ul
samples: PCR product 5uL + loading buffer 1uL (**E1-E2, C1-E4, E1-D3, D4-E4**) for check, or PCR product 18uL + loading buffer 2uL (**A1-A2, B1-B2, B3-B7**) for gel purification.

Gel photograph following “08252021”

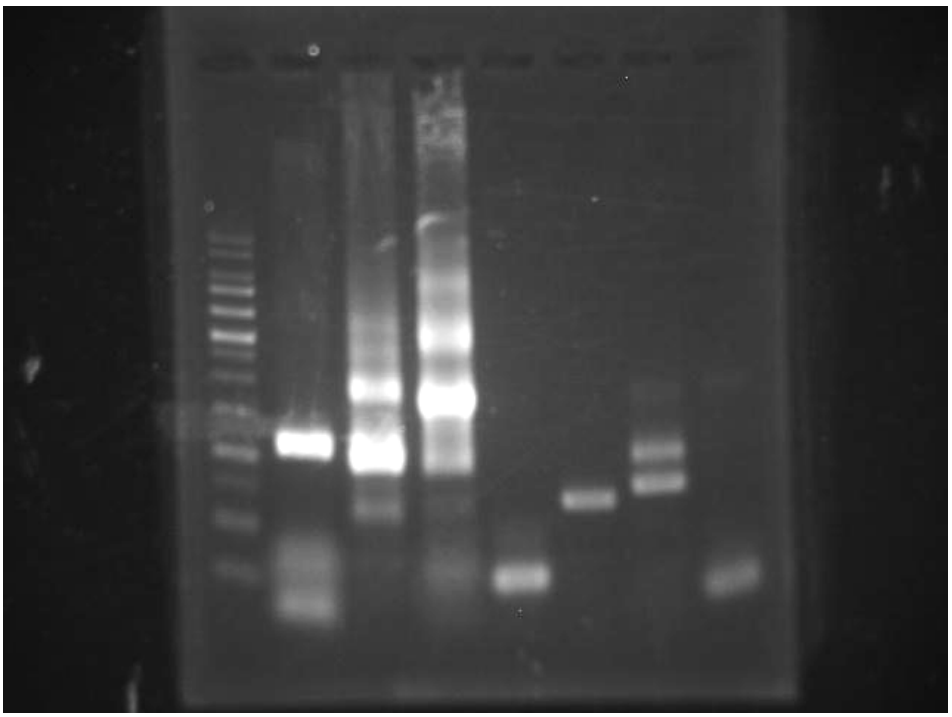
samples:

lame	sample	size	lane	sample	size
1	1kbp marker Marker	10-8-6-5-4-(3)-2.5-2-1.5-(1)-0.75-0.5-0.25 kb			
2	A1-A2 in plasmid A	1116	3	B1-B2 in plasmid B	1798
4	B3-B7 in plasmid B	1582	5	E1-E2 in plasmid C	271
6	C1-E4 in plasmid C	2523	7	E1-D3 in plasmid D	1765
8	D4-E4 in plasmid D	1953	-	-	-

08252021

#Production platform

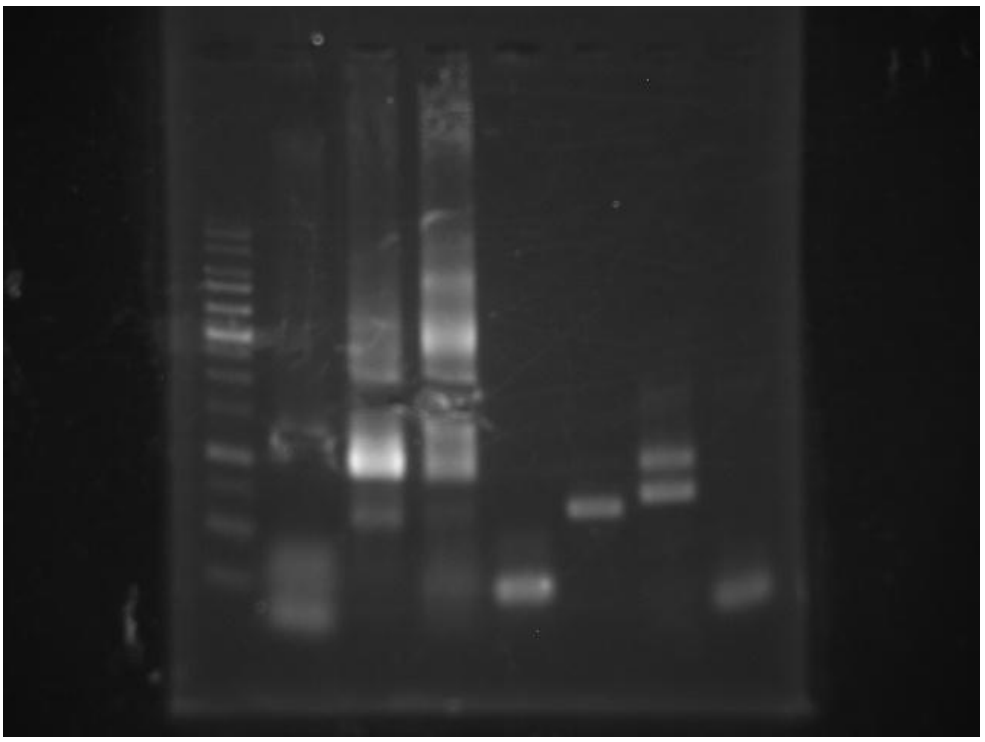
・Agarose gel electrophoresis (by Alex Liu)



In lane2~4 (A1-A2, B1-B2, B3-B7), the correct bands showed up.
gel-purify to isolate only the target bands.

It looks like the lane5 (E1-E2) has the correct band too, but the others do not.

after cutting:



gel purification by Wizard® SV Gel and PCR Clean-Up System (Promega).

conc.
A1-2: 12.2ng/uL
B1-2: 7.0ng/uL
B3-7: 21.8ng/uL

After gel purification, Alex used the above fragments and InFront Assembly for plasmid A and B cloning.

08262021

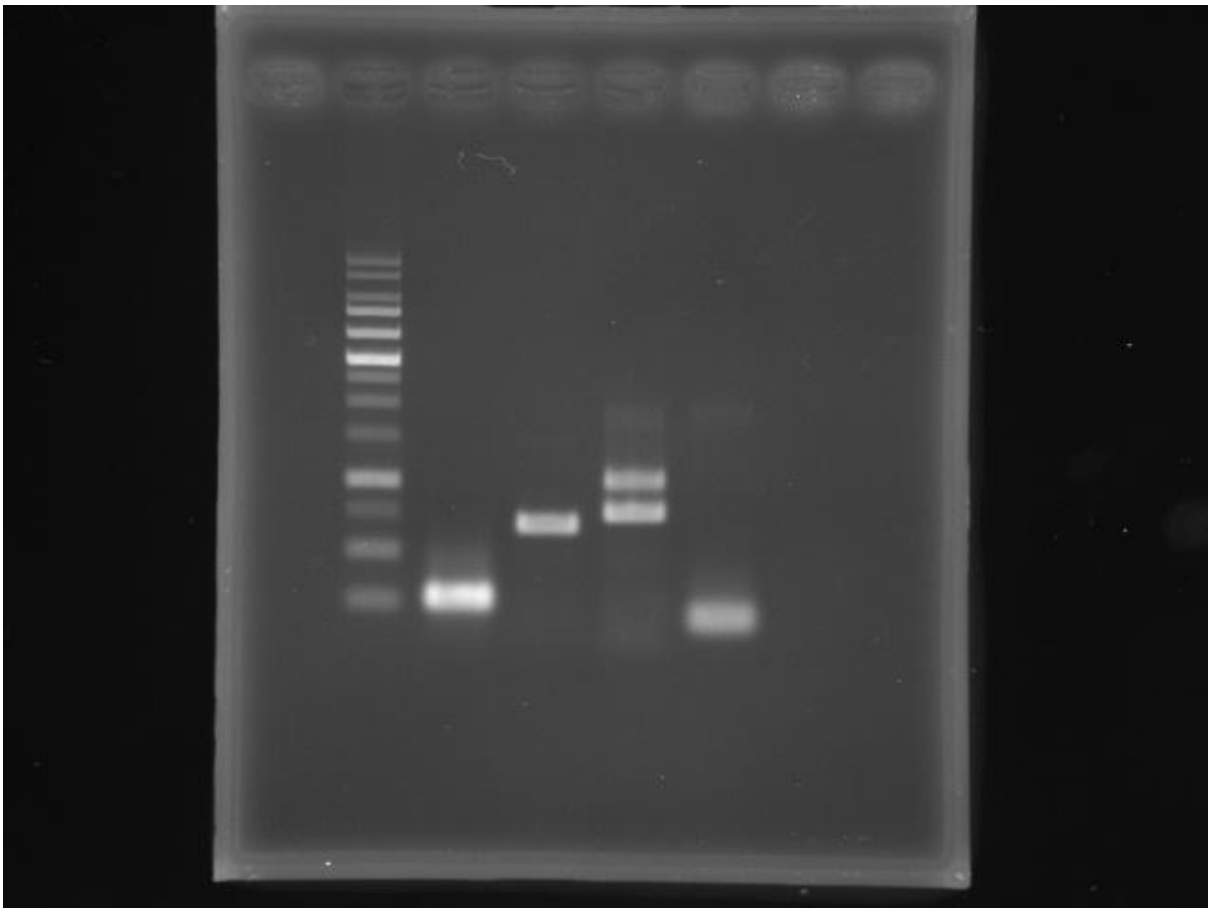
#Production platform

・Agarose gel electrophoresis

To confirm the length of C1-E4 and the band of E1-D3 and D4-E4.
run on 1% agarose gel for 25 min at 100V, then EtBr-stained for 30min.

marker: 5ul

samples: “08242021, OE-PCR” PCR product 5uL + loading buffer 1uL (**E1-E2, C1-E4, E1-D3, D4-E4**) for check.



samples:

lame	sample	size	lane	sample	size
2	1kbp marker Marker	10-8-6-5-4-(3)-2.5-2-1.5-(1)-0.75-0.5-0.25 kb			
3	E1-E2 in plasmid C	271	4	C1-E4 in plasmid C	2523
5	E1-D3 in plasmid D	1765	6	D4-E4 in plasmid D	1953

Lane 3, E1-E2, looks OK. Lane 4, C1-E4, is not completed and non-specific amplification happens on the band of about 700b. However, lane 5 and 6, E1-D3 and D4-E4, appear subtly. Especially in lane5, non-specific amplification happens on the bands of about 750b and 1kb.

・OE-PCR 003

The previous OE-PCR_08242021 shows fragments for plasmid A and B are completed, that E1-E2 is completed but C1-E4 is not for plasmid C and that the bands of two fragments, E1-D3 and D4-E4, appear subtly.

So, try OE-PCR amplifying E1-E2, C1-C2, E3-E4, D1-D2 and D3-D4 for plasmid C and D.

PCR program: **expected amplicon size = E1-E2 (271b)**

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

PCR program: **expected amplicon size = C1-C2 (759b), D1-D2 (774b), D3-D4 (939b)**

steps	℃	time	
predenature	98	1min	
denature	98	10sec	↑
annealing	**55**	15sec	30 cycle
extension	68	2min	↓
hold	16	∞	

PCR program: **expected amplicon size = E3-E4 (1794b)**

steps	℃	time	
predenature	98	1min	
denature	98	10sec	↑
annealing	**59**	15sec	30 cycle
extension	68	5min	↓
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 MasterMix	*	0.8	
PrimeSTAR GXL DNA polymerase		0.4	

total		20	

*

Template MasterMix	samples
E1-E2	**E1**(oligo_parS), **E2**(DT_1)
C1-C2	**C1**(TetR), **C2**(oligo_C)
E3-E4	**E3**(gB_lp-ecfp-parB_1), **E4**(gB_lp-ecfp-parB_2)
D1-D2	**D1**(TetR_082), **D2**(oligo_D)
D3-D4	**D3**(cl_2), **D4**(DT_3)

run on 1% agarose gel for 25 min at 100V, then SYBR-Safe-stained for 30min.
marker: 5ul
samples: PCR product 18uL + loading buffer 2uL (**E1-E2, C1-C2, E3-E4, D1-D2, D3-D4**) for gel purification.

Gel photograph following “08272021”

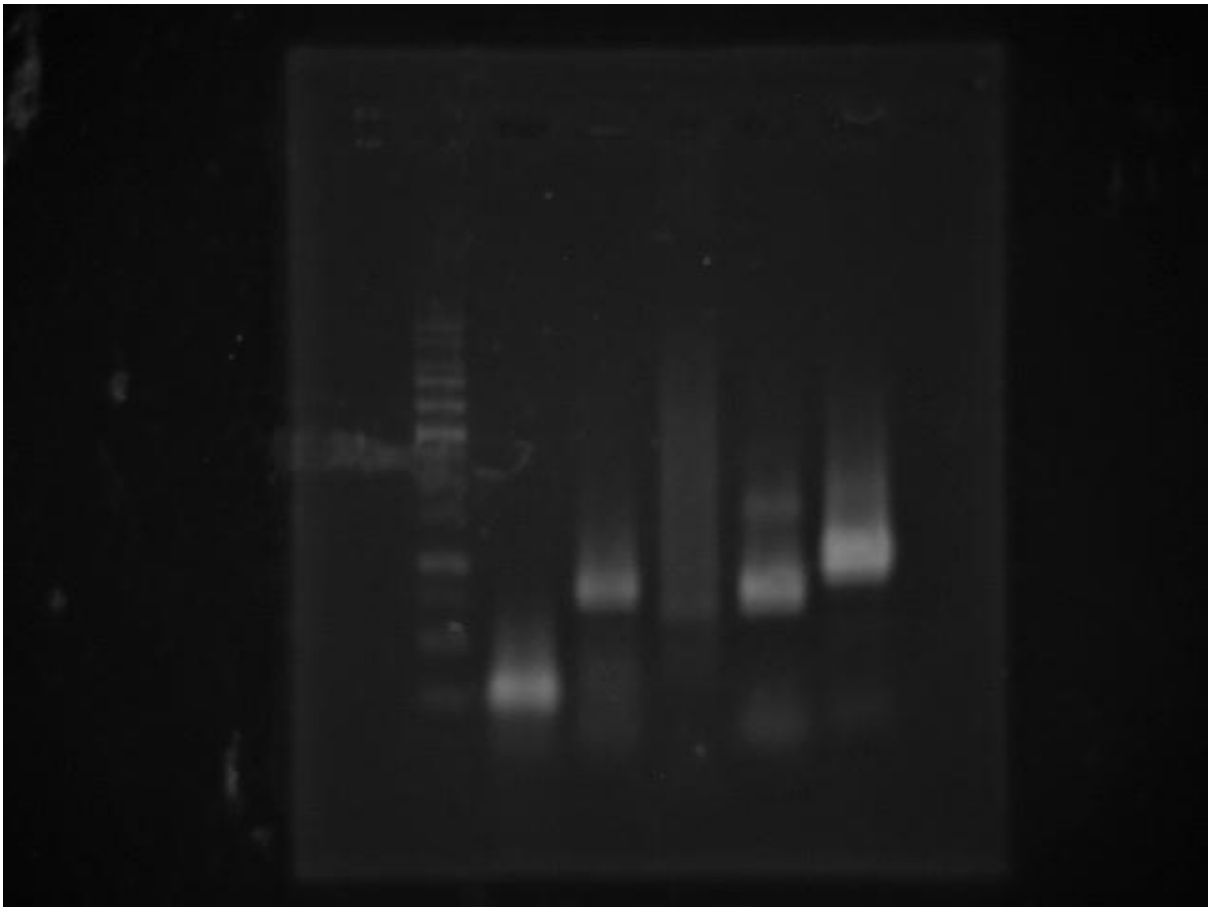
lame	sample	size	lane	sample	size
2	1kbp marker Marker	10-8-6-5-4-(3)-2.5-2-1.5-(1)-0.75-0.5-0.25 kb			
3	E1-E2 in plasmid C	271	4	C1-C2 in plasmid C	759
5	E3-E4 in plasmid C	1794	6	D1-D2 in plasmid D	774
7	D3-D4 in plasmid D	939	-	-	-

08272021

#Production platform

・Agarose gel electrophoresis

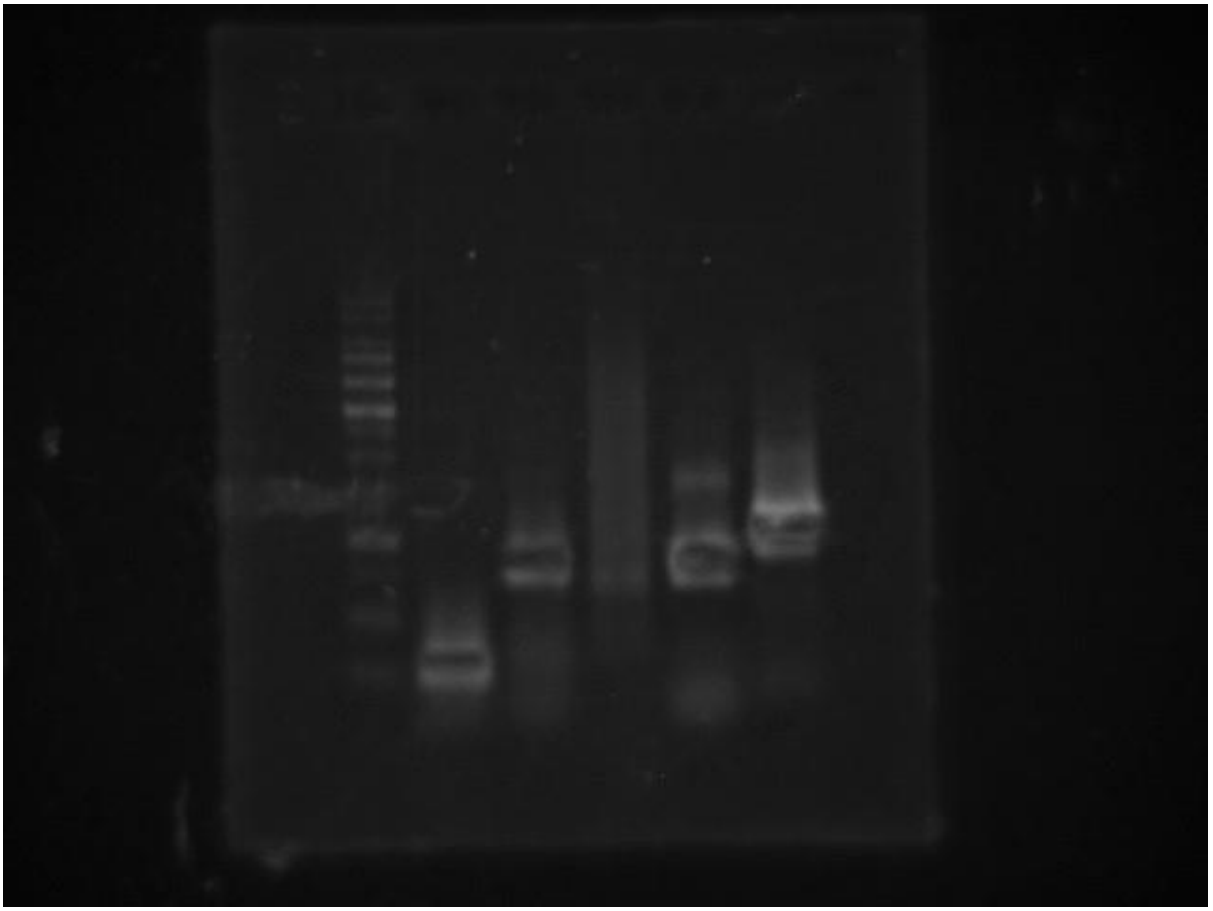
run on 1% agarose gel for 25 min at 100V, then SYBR-Safe-stained for 30min.
marker: 5ul
samples: PCR product 18uL + loading buffer 2uL (**E1-E2, C1-C2, E3-E4, D1-D2, D3-D4**) for gel purification.



lame	sample	size	lane	sample	size
2	1kbp marker Marker	10-8-6-5-4-(3)-2.5-2-1.5-(1)-0.75-0.5-0.25 kb			
3	E1-E2 in plasmid C	271	4	C1-C2 in plasmid C	759
5	E3-E4 in plasmid C	1794	6	D1-D2 in plasmid D	774
7	D3-D4 in plasmid D	939	-	-	-

Except lane 5, almost all lanes look OK. Lane 5 is very smeary and specific amplification doesn't happen on the lane. Considering this result and OE-PCR_08242021, it is hard to combine E3 with E4 for some reason.

after cutting:



gel purification by Wizard® SV Gel and PCR Clean-Up System (Promega).

conc.
E1-2: 15.0ng/uL
C1-2: 6.1ng/uL
D1-2: 13.1ng/uL
D3-4: 39.5ng/uL

Probably, using gBlock itself (E3, E4) instead of E3-E4, will do InFront Assembly for plasmid C and D.

08302021

#Production platform

・Colony PCR 003

reaction composition:

2x EmeraldAmp Max PCR Master Mix	40

MilliQ	36.8
f001 (F primer)@10uM	1.6
f002 (R primer)@10uM	1.6
total	80uL

for 15 samples:

IA 002	plasmid A	white 15 colonies (w11-w25)

PCR program:

steps	℃	time	
predenature	95	5min	
denature	98	10sec	↑
annealing	60	30sec	30 cycle
extension	72	5min	↓
hold	16	∞	

*to ensure cell lysis, predenature for 5min.

Gel photo following “08302021”

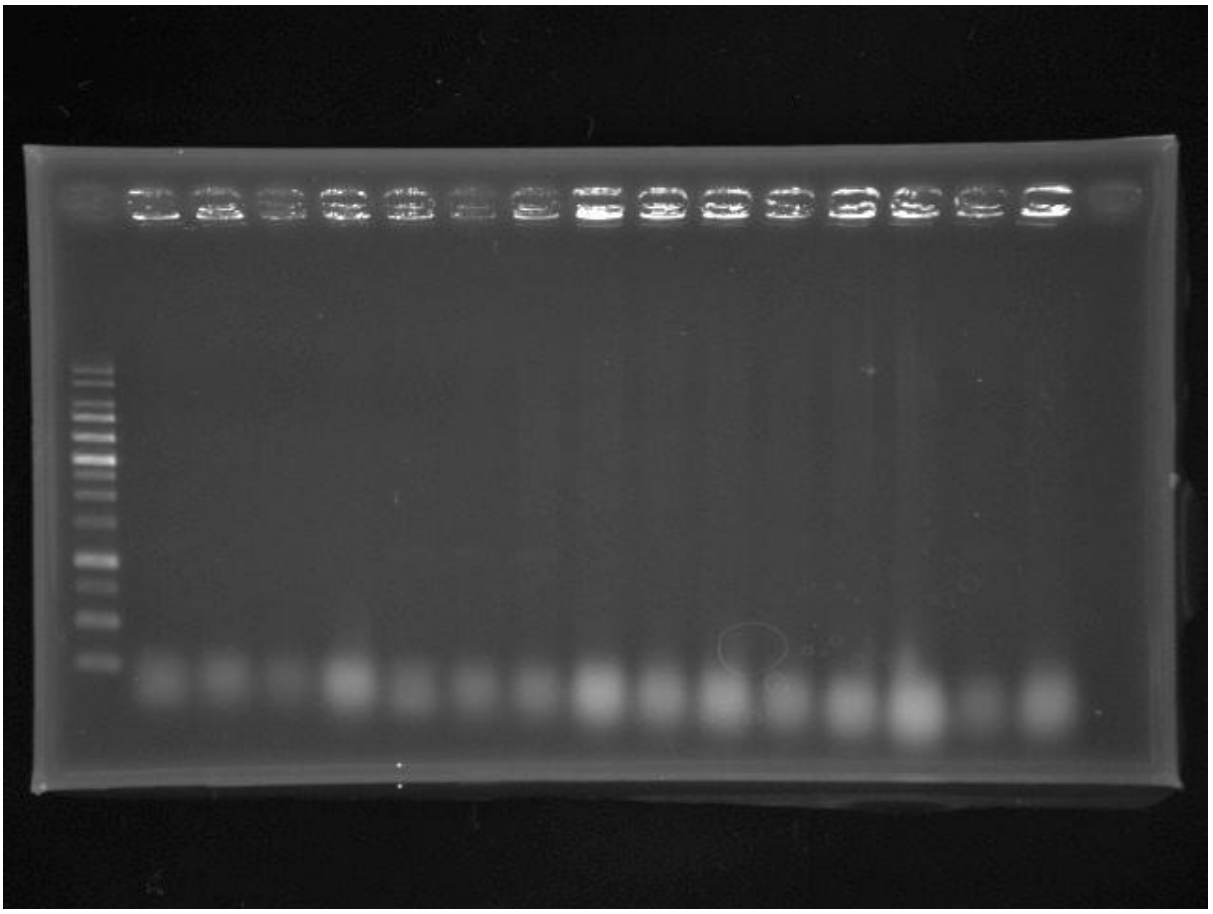
Marker: 1 sample, A (w11~w25)
Nothing looks correct because the desired band is 1875bp.

08312021

#Production platform

・Colony PCR 003 (following 08302021)

Gel photo following “08302021”



Marker: 1 sample, A (w11~w25)
Nothing looks correct because the desired band is 1875bp.
Maybe all of the plasmid A samples in IA002 failed.

・Miniprep and Measure concentration of plasmid B

Instead of Liu, miniprep the samples of plasmid B. Pick a colony to miniprep, so the names of samples are pSB4K5_B.

pSB4K5_B	51.8	ng/ul