Construct minimal *S. cerevisiae* mitochondrial genome

7.19- 8.9 in vivo

19th,July

PCR following fragments: 5-6 7-8 9-10 11 12-13 14-15 18-20

System: 10 μl (mix 5 μl F 0.4 μl R 0.4 μl T 0.4 μl H2O 3.8 μl)

Enzyme: PrimeStar max

program: Tm value 50 ° C extension temperature 62 ° C

20th,July

(1) Select Skeleton: Y33-ura

(2) Select strain: BY4741 (S.C)

(3) PCR following fragments: Fusion PCR 18-19+20

Re-prepare the first-stage fragment with miscellaneous bands and degradation: 1 3 4 6 7 10 12 13 15 16 17 20 21

System: 100 μl (mix 50 μl F 4 μl R 4 μl T 1.5 μl H2O 40.5 μl)

Enzyme: PrimeStar max

PCR program:9-10 gradient adjustment Tm value 52 ° C 54 ° C 56 ° C

(4) Results: 9-10 success; 16,21 fragment was unsuccessful

Condition: Tm value 56 ° C Extension temperature 66 ° C

21st,July

(1) PCR 16

Result: unsuccessful.

(2)Design primers of 2 LL series .

22nd,July

(1) PCR 11 and recycled.

(2) Preparation of reagents required for yeast lithium acetate conversion:

0.1M LiAc/TE Buffer

1M LiAc

DMSO

ssDNA

50% PEG3350

5Mm CaCl2

23rd,July

(1) PCR :fragment long 1 and recycled

System?

(2) Re-PCR 16

program:temperature rise and drop rate was 2 ° C / s

(3) Design verification excuses primer
(4) RePCR: L1-4
Template: 1-4 fusion PCR stock solution
Primer: NY22-1-F NY22-4-R
Enzyme: PrimeStar / i5 Control
Result: PrimeStar succeeded as i5 failed
(5) RePCR: 7-8
Template 7-8 Fusion PCR Stock Solution

25th,July
(1) Re-examine MT-16 plasmid
PCR in normal conditions
(2) Expand the fragment 16
PCR system and recycled.
(3) Fusion PCR: 2-3 5-6 9-10
(4) Re-prepared the first-level fragment with insufficient quantity: long 5 8
(5) fusion PCR :D12-13+D14-15
Primer: D12-F 13-R 14-F D15-R
program: 98°C /2min ,98°C /10s ,annealing rate 6°C/s to 72°C /1s, slow annealing rate 0.1°C/s to 40°C /15s， Extension temperature 62°C
(6) Re-PCR two skeletons: Y33-1, Y33-2
(7) PCR new LL primary fragments: LL1, LL3, LL4, LL6, LL7, LL9, LL10
Program: Tm value 50 ° C, extension temperature 62 ° C
System: 150 µl (mix 75 µl F 6 µl R 6 µl T 3 µl H2O 60 µl)
(8) Verify HK fragment: (list the correct results only) L1-4, 5-6, 6-7, 11, 12-13, 18-19, D4, D7, D18
(9) Verify HK 2-3 fragments.
Result: the distance from the correct strip position is about 300bp

26th,July
(1) Normal program PCR LL2
(2) Re-PCR: Normal 5, 8, LL5, LL8
System:10ul.
(3) Verify LLY331 LLY332 LL7-8 2-3* , 5-6*, 9-10* LL3
(4) Expand the primary fragment LL 1 LL4 LL6 LL7 LL9 LL3
System:150µl
(5) LL 10 gradient setting Tm value 52 ° C 54 ° C 56 ° C
(6) Verify recycling LL 1 LL4 LL6 LL7 LL9 LL3 LL Y33-2
(7) LL10 takes off 50°C×10, 55°C×10, 60°C×10
(8) Verify LL2 LL5 LL8 LL1-4
(9) PCR LL1-4
Result:1 LL 2 is correct, re-PCR after dilution

27th,July

(1) LL2 gradient set Tm value (52 ° C 55 ° C 58 ° C), run template control. If 55 ° C, 58 ° C was bright, would consider taking off
(2) Glue recovery LLY221 LLY332 LL3
(3) LL1-4 Increase Tm to 55°C, 58°C, 60°C, 62°C
(4) PCR LL5 LL8 (amplification)
(5)change downsteam primer of LL10 50 °C
(6) F9-10 not found
(7) transfer plasmid 5+6+7-8+9-10
(8) verify glue recovery LLY331 LLY332 LL3
(9) Fusion PCR: 9-10
(10) Fusion PCR 5-6, increase Tm value, gradient 55°C ,57°C, 59°C, 5-6 62°C expansion
(11) glue recovery LL5 LL8 measured concentration
(12) Re-PCR2 40°C 45°C 50°C
42°C 47°C 52°C
44°C 49°C 54°C

28th,July
(1) LL9-10 Verified and measured concentration
(2) Re-PCR L1-4
(3) Verify LL5 LL9
(4) PCR LL2-3 with LL2-3
(5) PCR ordinary 19
(6) Configure cm-ura solid medium
(7) Activated Saccharomyces Cerevisiae BY4741
(8) PCR LL5-6 system 150μl Tm value 59 ° C
(9) PCR :5-F LL-R 5-6L
System:10ul
Program: Tm 50°C
(10)PCR: 5-6+7-8
Program:Tm 55 chew 68 extension 62¨
PCR:7-8+9-10
Program:Tm 50 65
45 62

(11) Verified 5-6L L5-6 19 LL 2-3
(12) RecycledL1-4 LL5-6
(13) Amplification 19

29th,July
(1) glue recovery 19
(2) PCR: LL5-6
System:10μl
Program:59°C

(3) Bacteria verification did not succeed (true and false positive)
(4) Verify LL2-3 2-3 Error Strips
(5) fusion PCR LL5+LL6
System: f1: $2795 \times 0.04 / 72.7 = 1.54$ f2: $2288 \times 0.04 / 82.9 = 1.10$
Enzyme:Prime star: 5μl H_2O: 2.36μl
(6) Verify LL5+LL6 fusion stock, dispersion

30th,July
(1) Verified LL5-6 5-6
(2) PCR ordinary 17-18 f1:$1736 \times 0.04 / 126.1 = 0.56$ f2: $2370 \times 0.04 / 209 = 0.45$
Enzyme:Prime star
System:5μl; H_2O: 3.99μl
(3) colony verification
PCR dispensing verification
(4)PCR using the fused mother liquor LL5-6
Program: 52 ° C 54 ° C 56 ° C
(5) Verify 17-18 mother liquor
(6) RePCR normal 2,16,17-18
(7) amplified 16, 17-18
(8) Verified LL5-6 66°C LL5-6
Program:64 ° C

31st,July
(1) glue recovery 16, 17-18
(2) Gradient test P2 52 ° C 54 ° C 56 ° C
(3) PCR 5-6 with normal LL5+6
System ;f1:1.54μl f2: 1.10μl H_2O: 2.36μl
(4) Re-PCR 16
(5) PCR common 5 8
(6) Verified2 ,52 ° C 54 ° C 56 ° C, many miscellaneous bands
(7) glue recovery 16 5 8 no results
(8) Verify that MT-2 has almost no strips. Guess there is a problem with the template.
(9) Verify LL5-L6, dispersion error
(10) PCR using ordinary 5+6
System: f1: $2795 \times 0.04 / 81.4 = 1.37$μl f2: $2288 \times 0.04/102.9 = 1.10$μl
H_2O: 2.53μl
(11) RePCR and verified 8
System:10μl
(12) Enlargement 8
System:150μl
Program:0:45 50°C 62°C
(13) measured concentration 16, 8
(14) glue recycling 8

(15) Activated MT-2 glycerol

1st,August
(1) Extract MT-2 plasmid
(2) PCR 2
(3) Measured concentration 16 ,8 , 5-6 , 2
(4) glue recovery 5-6
(5) PCR LL5-6
Program:Repeat 59°C Change extension 62°C 64°C 66°C
(6) fusion PCR: LL5 + LL6 without slow annealing
System: f1=2795×0.04/70=0.160 f2=2288×0.04/82.9=1.10
(7) P LL2
(8) Verify MT-2 ,2 , LL5-6 , D5-10 , D7-10 þ 2 few hundred bp
(9) PCR LL5-6
Gradient:62°C 64°C 66°C
(10) DATEL D5-10, D7-10 Tm 52°C
Verification successful
(11) prepare LB solid medium
(12) picked bacteria BY4741, BY4743 K+
(13) PCR 2
System:150μl
Program: 50°C 62°C
(14) glue recovery ordinary α
(15) Verify LL5 + LL6 Fusion oroginal Solution, Dispersion

3rd,August
(1) conversed BY4743
L1-4+LLY33 1
LL5 + LL6 + LL7-8 + LL9-10 + LLY33 2
LLY33 1
LLY33 2
gd
sc

L1-4 9685×0.06/117.3=4.95 LLY33 1 4800×0.06/65.6=4.39
LLY33 2 4800×0.06/73.7=3.90 LL5 2795×0.06/70=2.39
L26 2288×0.06/82.9=1.65 LL7-8 2510×0.06/182=0.83
LL9-10 2829×0.06/127.6=1.33
(2) PCR LL11-LL21
System:10ul
(3) Prepare YPD
(4) Verify LL11-LL21, LL11-LL20, LL21
(5) PCR LL11-LL20
System:100ul

4th,August

(1) Glue recovery LL11-LL20þ
f1: 0.30μl f2: 0.62, μl

(2) Fusion PCR: 72-40 ° C slow annealing 14-15 þ LL14-15
System: 10μl
Verified successfully

(3) PCR small volume LL12-13 LL17-18 LL18-19þ
Verified successfully

(4) PCR LLY33 3 LLY334 LLY33n1

(5) PCR large volume LL12-13 LL17-18 LL18-19
System:150μl

(6) glue recovery LL12-13 LL17-18 LL18-19þ

(7) RePCR: LL16
System: 150μl
strip was dark

(8) Measured concentration LL11-LL20 LLY33 LLY33 LLY33n1 LL12-13 LL17-18 LL18-19

(9) PLL16þ Purification

(10) PCR Ln1-4 LLn1-4 L21 LL21

(11) PCR D18-20 → L18-20 D16-17 → L16-17 D19-20→L19-20
Program:50°C 62°C

(12) Verify Ln1-4 ,LLn1-4,L21 þ LL21

(13) PCR large system LL14-15

(14) glue recovery LL14-15
no correct strip

(15) PCR LLn1 þ Glue recovery

(16) point gel verification enzyme digestion 2; few hundred bp smaller

(17) Enzyme-cutting bacteria: 1-3-1 2-2-3 2-2-4

(18) Fusion Normal 18-19+20
Program:72-40°C slow annealing ;No purpose strip
19+20 72-40°C slow annealing

(19) Verify HKL1-4 Availability

5th,August

(1) PCR system L21 Small system LL14-15

(2) Measured concentration LLn1 LL12-13 LL17-18 LL18-19 LL161 LL162

(3) Verified enzyme digestion 1-3-1 2-2-3 2-2-4 LL161 LL14-15 (rePCR version)

(4) Conversion BY4743 Starting at 114:00 Starting at 219:00 (Debug DMSO)
9.80μl 3.74μl
1-1 L1-4 + LLY33 1
1.26 0.61 0.61
1-2 LL1 LL2 LL3 LL4 + LLY331
1.26 0.61

1-3 LL1 + LL2-3 + LL4 + LLY331
2.39μl 1.65μl 0.83μl 1.33μl 3.66μl
2-1 LL5 + LL6 + LL7-8 + LL9-10 + LLY33 2
0.64μl 1.43μl 0.87μl 0.37μl 1.42μl (black)
3-1 LL11 + LL12-13 + LL14 + LL15 + LL16 + LLY33 3
4 LLY331 3.74μl
5 LLY332 3.66μl
6 LLY333
7 gd plasmid
8 Sc

(5) Took a look at the overgrown bacteria, is it sputum?
(6) Fusion α-3 f1=2265×0.04/30.8=2.94μl H2O 1.52μl
7α-40°C slow f2=2883×0.04/202=0.54μl
(7) Fusion LLn1 + LL2-3 + LL4 f1=2778×0.04/156.7=0.71μl
s: 75-40 ° C slow F: 98-40 normal f2 = 5148 × 0.04 / 41 = 5.02 μl
F3=1759×0.04/114.5=0.61μl
(8) purified α1þ
(9) Measured concentration LL2 LL2-3 Lα1 Ln1þ
(10) large system LL14-15 LL2 LL2-3 PCRþ
(11) glue recycling LL2 LL2-3þ
(12) Verify D12-15 → L LLY333 LLY33 n1 LLY33 4 LL14-15¨
(13) Purification LL14-15¨
(14) Fusion 12-13+14-15

6th,August

(1) A1 + LLn1 melt s: 72°C-40°C / F 72-40°C
F1=525×0.04/46.3=0.45μl f2=2778×0.04/156=0.71μl
(2) Expand D12-15
(3) Many small systems P 14-15 melt first
(4) Verify α1
(5) 1.5-level carrier þ , with Y33 Vector 10μl
(6) Purification LL2-3 2-3þ LLY334
(7) Gradient Tm D12-15 → L 54°C 56°C 58°C
(8) PCR LLn1-4(s/F) 10μl þ
(9) PCR LL2-3 (50μl) 2-3 (50μl)
(10) PCR LLY334
System:50ul
10μl LLY333 57°C 59°C
10μl LLY33n1 57°C 59°C
(11) Expand PCR 1.5 class carrierþ
(12) rePCR n21 þ verificationý
(13) glue recovery LLY333 LLY334þ

7th,August

(1) Glue recovery P(7-8) P(12-15) P(18-19) L(12-15) LLY333 LLY334
(2) RePCR n21
gradient 35 40 45
45 50 55
(3) measured concentration LLY33 3 not available
(4) Verified the new LLY33 n1 success Expand PCR
(5) Purification 90μL LLY33 3 LLY33 n1 LLY33 4
(6) Prepare 1M LiAc CoCl2 TE Sterilize ddH2O
(7) conversion 0.1×
1-1 PH: L1-4 24.5/8.26 LLY33 1 9.35
1-2 P1-2: LL1 3.15 LL2-3 12.56 LL4 1.53 LLY33 1 4.41*
1-3 P1-3: LL1 3.15 LL2 1.21 LL3 1.68 LL4 1.53 LLY33 1 4.41*
2-1 P2-1: 5-6 9.68 LL7-8 2.08 LL9-10 3.33 LLY33 2 9.15
2-2 P2-2: LL5 5.98 LL6 4.13 LL7-8 2.08 LL9 1.56 LL10 1.82 LLY33 2 1.72*
3-1 P3-1: LL11 1.6 LL12-15 18.26 LL16 3.55 LLY33 3 5.50
3-2 P3-2: LL11 1.6 LL12-13 3.58 LL14-15 5.80 LL16 3.55 LLY33 3 5.50
1-0 P3-0: LLY33 3 5.50
4 gd:
5 sc:
(8) RePCR L1-4 LL2-3 5-6 LLY33 1 LLY33 2 LL5 L12-15
(9) Fusion L1+2-3+L4 f1: 2778×0.04/89.1=1.25
F2: 5148×0.04/35.9=5.73
F3: 1759×0.04/136=0.52
5+6 f1: 2795×0.04/81.4=1.37
F2: 2288×0.04/102.9=0.89
H2O: 2.74
(10) measured concentration LLY33 1 LLY33 2
(11) Glue recovery LL2-3 LL5

9th,August
(1) Verified 7 → 4 = 28 × α A priori
(2) DH5α 1.5 grade 8μL+100μL transformation
6μL running glue
LB A+
(3) Conversion
1-1 PH: L1-4 10.96 LLY33 1 4.41
1-2 P1-2: LL1 3.15 LL2-3 4.32 LL4 1.53 LLY33 1 4.41
1-3 P1-3: LL1 3.15 LL2 1.21 LL3 1.68 LL4 1.53 LLY33 1 4.41
2-1 5-6 6.66* LL7-8 1.20 LL9-10 3.33 LLY33 2 1.72
2-2 LL5 2.97 LL6 4.13 LL7-8 1.20 LL9 1.56 LL10 1.82 LLY33 2 1.72
3-1 LL11 1.6 LL12-15 7.09 LL16 3.55 LLY33 3 5.50
3-2 LL11 1.6 LL12-13 3.58 LL14-15 5.8 LL16 3.55 LLY33 3 5.50
1-0 LLY33 1

2-0 LLY33 2
4 gd
5 sc

19:00 Transformation, and then Coating plate
Carry on nucleic acid electrophoresis

2018.8.6
9：00 PCR again ATP6(primestar
20μl primestar 10μl
F. R 0.8μl
Template 0.4μl
Water 8μl
Annealing(55℃) Extending（72℃）

Verification by nucleic acid electrophoresis：
ATP6-6 and COB V-1 The location of the strip is correct and bright.
COB V-8 The strip isn’t showed in the figure.

PCR again VAR1 Primestar 61℃， 62℃ 20μl
enzyme 10μl
FR 0.8μl
Template 0.4μl
Water 8μl
Verification by nucleic acid electrophoresis：
The location of the strip is correct and bright.

PCR VAR1 Primestar 61-62 200μl
Verification by nucleic acid electrophoresis: Rubber recovery, concentration: low.

2018.8.7
Extract plasmid of ATP6, measure its concentration.
Colony PCR ATP6 T5 super mix 20μl 2×4

Super(enzyme)	10	40	-19.6
F.R	0.8	3.2	
Bacteria		0.4	
H_2O	8	32	

Carry on nucleic acid electrophoresis：no strips.

PCR：VAR1 200μl Primestar mix
Primestar 100μl
F.R 8μl
plasmid（7.31） 4μl
H_2O 80μl

Carry on nucleic acid electrophoresis：no strips.

Carry on nucleic acid electrophoresis of the plasmid and segment of VAR1.
(verification of 20180807-VAR1)
①The first plasmid（7.25）
②The first segment
③④The second plasmid
⑤⑥Reconnect plasmid

2018.8.8
PCR
Plasmid COX1 COX2 COX3 ATP8 ATP9 ATP6 1.2 COB VAR1 IC3 ATP6 1.2
Segment COX1 COX2 COX3 ATP8 ATP9 ATP6 1 COB VAR1 IC3
nucleic acid electrophoresis：The location of strips are all wrong.

2018.8.9
PCR and then verification the segment. Result: All are wrong except COX1 and COB.
Activation ,extract plasmid and measure the concentration.
ATP9 VAR1 ATP6 COX3 IC3 ATP8 COX2 COB COX1
COX1 125.8
COX2 172.9
COX3 204.2
ATP8 85.78
ATP9 321.2 (×)
ATP6 50.59
COB 55.39
VAR1 106.8
IC3 126.4

2018.8.10
PCR: I5 10μl
COX1 COX2 COX3 ATP8 ATP9 ATP6 COB VAR1 PSBIC3

2018.8.10
PCR { I5 ATP8，ATP9，IC3
Primestar COX1 IC3 }
Carry on nucleic acid electrophoresis of COX1 COX2 COX3 ATP8 COX3 COX2 COX1 IC3
All locations are correct and bright.

10μl {
xbaI： 0.2μl
CutSmart： 1μl
plasmid： 200ng
H_2O：
}

37°C，3h

		V	VH_2O
psBIc3	79	2.53μl	6.27
COX1	81	2.47μl	6.33
COX1	177	1.13	7.67
ATP9	36	5.6μl	3.2

2018.8.11

Colony PCR， I5 98°C， 20μl

COX2 2 3 4 COX3 2 3 4 (shallow) COB 2 3 4 ATP6 2 3 4

COX1 2 3 4 VAR1 2 3 ATP8（have） ATP9（have）

	CPEC	C	V		L	H_2O	
	IC3	74	2098	4.196	2070		
2850 *	ATP6	67	0.873	1.746	78.0	44.058	44.
2826	COX2	70	0.81	1.62	75.6	44.184	44.2
2880	COX3	54	1.125	2.25	8.10	43.554	43.6
3228	COB	58	1.498	2.996	11.58	42.808	42.8
3267	VAR1	55	1.633	3.266	11.97	42.538	42.6

Make LB solid medium (C+) 16

ATP6: carry on CPEC , transformation and coating plate.

Carry on amplification of segment VAR1， PSBIC3， COX3，COB

Nucleic acid electrophoresis results: no strips, no strips, bright strips

PCR pSB1c3 again, and then do nucleic acid electrophoresis. The strip is a little bit thin, and the concentration of segment after gel recovery is 31.57, while the concentration of plasmid after gel recovery is 97.35.

The concentration of COX1 after gel recovery is 105.1, and the concentration of COB after gel recovery is 53.04.

	C	25μl	50μl	50μl
COX1	105	0.58	1.16	47.24
COB	53	1.14		21.76
IC3	97	1.6		

2018.8.13

12:00 Carry on CPEC：COX2 COX3 COB VAR1

16:00 Transformation

13:00 Verification ATP6 by colony PCR， I5 . 55°C/72°C，（9）

Carry on nucleic acid electrophoresis, but no strips.

17:30 Coating plate.

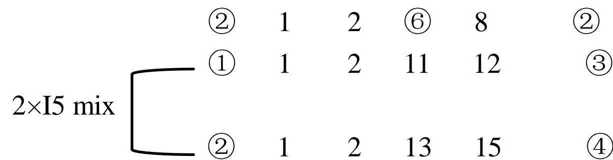
21:00 Activate CPEC-ATP6.

2018.8.14

9:30 Colony PCR primer：①Normal segment ②Sequencing primers

20μl ① 1 2 5 7 ①

2×T5 super mix



12:00 Nucleic acid electrophoresis， T5-②-6 18 exist strips（but a little bit bigger than expected）

I5-①-③ strips exist at the bottom（a little bit small）

11:30 Activate ten of each COX2，COX3，COB，VAR1.

19:00 Colony PCR

	T5						I5			
	1	2	3（II）	4	5	6（CX）	7	8（II）	9	10（CX）
COX2	√	√	√ (不对)	√	✖ √	☑	☑			
COX3	☑	√	√	√			☑	√		√
COB	☑			√	小	√				
VAR1		☑		☑						

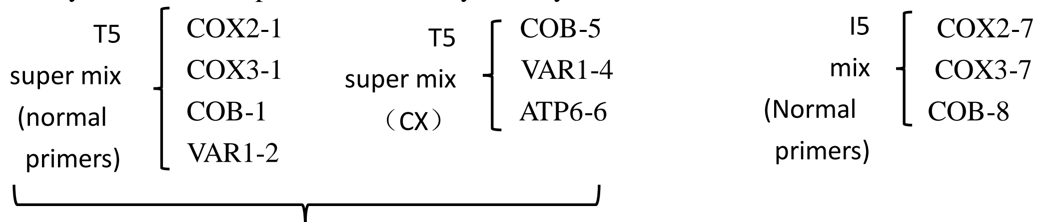
22:40 Carry on nucleic acid electrophoresis, but the results are all smaller than expected.

2018.8.15

Extract plasmid of COX1-2， COX2-7， COX3-1， COX3-7， COB-1， COB-5， COB-8， VAR1-2， VAR1-4, which has showed strips in colony PCR yesterday.

2018.8.16

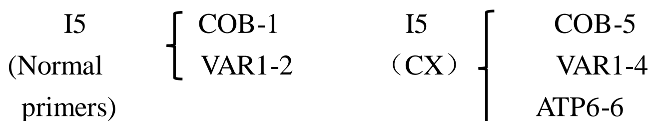
Carry on PCR of the plasmid extracted yesterday.



All wrong location

COX2-7 Correct location, and bright strips.
COX3-7 No strips.
COB-8 Correct location, but not bright.

Exchange enzymes and PCR again.



The results of nucleic acid electrophoresis: COB-1 has correct location and bright strips, while others are all failed.

Activate ATP8, ATP9, VAR1-6, ATP6-6（We are going to sequence these genes.）

2018.8.17
Extracting plasmids.

9.7
The plasmid of VAR1 degraded.

PCR	PSB1C3		PSB1C3-F	F	2		
			PSB1C3-R	R	2		
		50μL		T	0.5	ATP-9	55
				Mix	25		
				H2O	20.5		

P	V.S	10μL	vu-F(60)	F	0.4		
			vu-R(60)	R	0.4		
				T	0.5	Y33 vector	Primer star
				Mix	5		
				H2O	3.7		

Tm: 57 59
Tm succeed at 57, but strip is a little bit lower.

9.8

Carry on PCR of VAR1	10μL	1C3-VAR1-R	F	0.4	
		1C3-VAR1-F	R	0.4	
			T	0.5	T: MT-11 VAR1
			Mix	5	
			H2O	3.7	

VS.59 correct strips, a little bit trailing. Then run the electrophoresis bath at low voltage again, but still trailing.

PCR	V.S. 60	10μL	vu-F(60)	F	4
			vu-R(60)	R	4
				T	1
				Mix	50
				H2O	41

VAR1 has been enlarged by PCR,and we found that Tm 59°C is better than Tm 60°C.
Then we enlarged the system, and purification.

50μL	1C3-VAR1-F	F	2
	1C3-VAR1-R	R	2
		T	1
		Mix	25
		H2O	21

Carry on CPEC	VAR1	1197bp	89.775ng	×2	179.55ng	1.81μL
	PSB1C3	2070bp	155.25ng	×2	310.5ng	3.86Ml

VAR 1.81
Desired mix 50μL PSB1C3 3.86 →reaction 20μL 10μL primer star
H2O 44.33 10μL Desired mix

9.9
Mix 5
F 0.4 CC33-1-F
PCR segment 1 from 21-1 10μL R 0.4 CC33-L1-R
T 0.5 21-1 from refrigerator
H2O 3.7

PCR 9-10 again 10μL LL33-9-F T: LL9-10 ①
11-10-R LL9-10 ②

PCR 9-10 again 10μL LL33-9-F F: LL9-N10
11-10-R
D12-15 → D12-15 10μL D12F(D11-12-F) T: D12-15 （run out）just a little
D15R(D16-15R)

☆L12-15 → L12-15 10μL 11-12-F T: L12-15 Tm: 52
16-15-R
L12-15 → LL12-15 10μL LL33-L12-F T: L12-15
LL33-L15-R
×3 Tm: 50 52 54☆ 30μL Mix 15
F 1.2
R 1.2
T 1
H2O 11.6

PCR segment 1 from 21 :correct location, but smaller 100bp than 21-1.
PCR 9-10 again. ①② no strips.

5-8 10μL ×6 4-5-F 228① 5-81
L-8-R 228② 5-82
Mix 5×7=35 227 6K 5-83
F 0.4×7=2.8 227 12K 5-84
R 2.8 9.5 227① 5-85
T 0.5 227② 5-86
H2O 3.7×7=25.9

9-10 exists correct strips. But there are two strips at 2800bp,and some incorrect strips exist at small location.(suggestion: increase Tm)
LL12-15 strips are strange. And 52 is better than 50.

L12-15 succeed.But there is a stray band at 4000bp. It's necessary to increase Tm.
5-8 1、2、5、6 exist correct band，2 is brightest，3、4 no strips.
228①、228②、227①、227② can use later，2276k 和 22712k can't use later.
LL-9-n10 2、3 correct band(a little dispersion)，stray bands are a little bit strange，2 is better than 3，3 is more disperse and less bright.

9.14

VAR1 not succeed last time,measure the concentration of pSB1c3 and found that is very low.

	VAR1	2μL
CPEC	PSB1C3	5.02μL
	H2O	42.98μL

MT-9	ATP9	231bp
MT-4	ATP8	147bp
MT-4 MT-5	ATP 6	780bp
MT-6 MT-7	COB	1158bp

9.15

PCR	PGF	F	0.4	PGF1	21-PGF-R(90)
		R	0.4		GFP-PGF-F(90)
	10μL	T	1	PGF2	21-PGF-R(60)
		Mix	5		GFP-PGF-F(60)
		H2O	3.2		Tm: 55 57

ATP8	1C3-ATP8-F	147bp
(MT4)	1C3-ATP8-R	
ATP9	1C3-ATP9-F	231bp
(MT9)	1C3-ATP9-R	
COB-u	1C3-COB-F	415bp
(MT6)	COB-MT6-R	
COB-d	COB-MT7-F	780bp
(MT7)	1C3-COB-R	
ATP6-u	1C3-ATP6-F	353bp
(MT4)	ATP6-MT4-R	
ATP6-d	ATP6-MT5-F	519bp
(MT5)	1C3-COB-R	

PGF succeed (55 is better),enlarge the system.

	F	4
	R	4
100μL	T	10
	Mix	50

H2O 32

CPEC ATP6 desired mix PSB1C3 155.25ng/61.8=2.51 5.02
ATP6d 38.925ng/21.8=1.79 3.58 H2O: 35.1
ATP6u 26.475ng/8.4=3.15 6.3

ATP8 PSB1C3 155.25ng
ATP8 11.025ng/15.2=0.73 1.46 H2O: 43.52

ATP9 PSB1C3 155.25ng
ATP9 17.325ng/23.0=0.75 1.50 H2O: 43.48

COB desired mix PSB1C3 155.25ng
COB u 31.125ng/16.6=1.88 3.76 H2O: 32.86
COB d 58.5ng/14.0=4.18 8.36

9.16

Bacteria grew on Cmr. So Cmr is correct.

100μL PSB1C3 PSB1C3-F VAR1 1C3. VAR1. R
PSB1C3-R 1C3. VAR1. F

Maybe the segment is not pure.

100μL F 4
R 4
T 0.5
Mix 50
H2O 41

10μL verify if the primers are correct. F 0.4
R 0.4
T 0.5
Mix 5
H2O 3.7

PGF·real·plasmid 10μL F 0.4
R 0.4
T 1
Mix 5
H2O 3.2

PGF GFP-PGF-F(90) PGF(H1) 11-PGF-F(90) PGF(H2) η-(H2)-PGF-R
21-PGF-R(90) 21-PGF-R(90) GFP-PGF-F(90)

21 PGF-21-F(90) 150μL F 6
2233-21-R R 6
T 0.5
Mix 75
H2O 62.5

55、72、PGF are all succeed but have stray band，we are going to increase Tm.(H2 has less stray

band)

9.22

150μL enlarge PGF1 F 6
R 6
T 3 H1 H2
Mix 75
H2O 60

21 F 0.4 21A 1μL A template rest are 0.1×21
R 0.4 21Fly 21Fly are as normal 21Fly +5: 40 45 50 fly PCR，58extending\failed
T 1 21lard normal P 50 +5：53
Mix 5 21nomdl 50：40、45、50 fly PCR，58 extending 52：45、50、55 fly PCR，
H2O 3.2 62 extending \failed
21Fly+CPEC 55℃ normal 10 recycle OPEC 10 recycle

21-①，maybe too little
LLn1 √
☆EN2 thin but is correct. 2 bands,one of them are about 2000bp (brighter)，another is the same as LL6.(less bright)
3-N-4 √ 4642
A4-N5 √ 4554
☆ LL6①√ but strong dispersion
LL6② A band which is less than 2000bp has dispersed to 22kbp.(may degrade)
NL5 √
☆N5-8① 7593 √ a little degrade
☆N5-8② √ dispersion
7-8 2510 √ dispersion up and down,too many stray bands.
LL9-n10 2829 correct band, but have strong dispersion.(gel recovery)
LL9① √
LL9② √
NL10 √
N11 √
N11-12 √ 4281
NL11-12，② √
☆LL11-H1 √ should be 1904bp, but is about 1300bp showed in the figure.
☆12-13 √ but very little left 3753
☆LL14-15 √ 2925
LL12-15 Two bands(one 6000bp,one 3500bp).Correct should be 6000bp.But 3500bp one is bright.
15-N16 √ a little dispersion，should be thin，exposure 1000

N17-18 √ 4133
LL18-19 √ 5676 almost
20-GFP⑤ almost
☆LL11-H2 √ Two bands,one just more than 1000bp(bright one),another more than 2000bp(very shallow)
PGF H1/PGF H2 only once

9.23
n5-8 150μL F 6 4-5-F
R 6 L-8-R
T 3 228①
Mix 75
H2O 60
n5-8 dimer and 12-13 dimer is a lot.
test the condition.
10μL F 0.4 n5-8 4-5-F 1.2
R 0.4 L-8-R ×3 1.2
T 0.4 T 228① 1.2
Mix 5 n5-L8 4-5-F ×3 15
H2O 3.8 LL33-L8-R 50、52、54 11.4

15:00 0D: 7-9 （10+x）0.1=7.9x
LL12-13 1、2 both exist，1 is brighter

n5-8 fail to exist n5-L8 all exist, same brightness，54°C is more bright, maybe L-8-R go bad.
150μL enlarged system F 6
R 6 4-5-F
T 3 LL33-L8-R Tm 54
Mix 75
H2O 60

Standardization

2018.7.16
08:30 Preheated instruments for 30min
09:00 Diluted the bacteria solution to 1:8, and measured concentration
09:25 Diluted to 0.1, measured again

	NC1	NC2	PC1	PC2
Before dilution	2.728	2.963		2.731
After delution	0.312	0.389		0.321
	0.328	0.372		0.349

	0.383	0.35		0.349
average	0.126	0.127		0.124

09:45 Diluted the bacteria solution 5 times following instructions
10:10 Inoculated the 3rd, 4th, and 5th dilutions of NC1, NC2, PC1, and PC2 on the medium, applied evenly, and refrigerated.

2018.7.18
36 medium colonies

	NC1	NC2	PC1	PC2
D3	416	380	436	464
	420	432	480	512
	680	368	468	496
D4	88	52	50	50
	74	52	46	72
	65	44	51	61
D5	7	2	5	6
	8	6	5	9
	9	5	7	7

7.25
10:00
Activated MT3, 4, 9, 11, 19, 20
System: 5 ml of LB liquid medium, 5 ul of resistance (Amp, K) (1:1000), 50 ul of glycerol solution (1%).
Cultured: 37 ° C, 220r.
19:00
Preserved glycerol bacteria: 1 ml bacterial solution, 500 ul 50% glycerol, and stored at -80 °C.
20:00
Extract six glycerol bacteria plasmids activated in the morning, and the plasmid concentration was measured, as shown in the following table:

No.	MT3	MT4	MT9	MT11	MT19	MT20
Concentration	165.3	209.2	34.8	88.04	41.18	94.41

Stored at -20 °C.

2018.7.26
9：00
500 ml of LB solid medium was prepared, sterilized, cooled, and resistant chloramphenicol C+ was added.

11:00
The PSB1C3 plasmid was transformed into BL21-DH5α competent cells: competent 50 ul, plasmid 2 ul.
12:00
The plate was incubated in a 37 ° C incubator.

2018.7.27
9:00
The concentrations of MT9 and 19 were low .
Reactivated system:5 ml LB + 5 ul resistance.
The plate was grown with a single clone yesterday, and picked the PSB1C3 monoclonal activation.
20:00
The PSB1C3 glycerol bacteria were prepared.
Extracted the PSB1C3 plasmid and the concentration was measured.

2018.7.28
8:30
Activated MT4, 5, 6, and 7.
18:30
Diluted the primers.
PCR
System:10ul
enzyme:I5,2×I5 mix 5ul
Primer F, R 0.4ul
H2O: 4.2ul
Template: a little
PCR program: 98 ° C 2mix
98°C 10s
34×
(annealing temperature) 55 ° C 15s
(extending temperature) 72 ° C 15s / k
72°C 5min
12 ° C

Gene	COX1	COX2	COX3	ATP8	ATP9	ATP6	COB	VAR1	PSB1C3
Length	1605bp	756bp	810bp	147bp	231bp	780bp	1158bp	1197bp	2070bp

2018.7.29
8:30
Run the nucleic acid electrophoresis test (except for VAR1) all have strips and the position is correct.
PCR All fragments except VAR1, 100 ul system, I5 enzyme.

10:30
Run the nucleic acid electrophoresis test, all the strips and the position were correct.
Glue recovery and the concentration was measured: COX3: 54.22. Remaining concentrations were all low, discarded.
14:00
PCR conditions for VAR1: annealing-extension: 50/52/54/56-72, 50/54/56-62
15:00
Run the nucleic acid electrophoresis test, no bands.
20:00
RePCR: ATP6U ATP6D ATP8 ATP9 COX1 COX2 COBU COBD PSB1C3

2018.7.30
8：30
Glue recycling yesterday, the concentration measured is shown in the following table:

No.	ATP6U	ATP6D	COBU	COBD	COX1	COX2	COX3	ATP8	ATP9	PSB13
Concentration	34.31	77.25	114.3	153.6	44.61	70.98	54.22	36.27	24.41	74.12

Fusion PCR: ATP6, COB
Step1
fusion: 10ul system 55°C, 15×, extension 30s
ATP6 diluted 5 times I5 mix 5ul, ATP6U 2.2ul, ATP6D 1.24ul, H2O 1.6ul;
COB diluted 10 times I5 mix 5ul, COBU 1.5ul, COBD 2.1ul, H2O 1.4ul.
20ul system: I5 mix 10ul, primer F, R 0.8ul, product 2ul, H2O 6.4ul.
After running the nucleic acid electrophoresis, the COB strips are bright and in the correct position, and the ATP6 strips are dark and the bands are bright.
Step2
Amplification: 200ul system 55°C, 35×
I5 mix 100ul, primer F, R 8ul, first step product 4ul, H2O 80ul.

2018.7.31
8:30
Re-extracting VAR1 plasmid
PCR 10ul system Robust fusion enzyme: 55/58/60/61/62-72, 45s, 25×
5×buffer 2ul
dNTPs 0.2ul
Positive and negative primers 0.5ul
Template 0.2ul
H2O 6.5ul
RT polymerase 0.1ul
Ran nucleic acid electrophoresis, no bands.
10:30
COB, ATP6, and COX3 grew colonies, and the rest didn’t. (one-step cloning)

COB, ATP6, COX3 , picked 6 single colonies to activate in 2ml EP tubes, 37 ° C, C+
RePCR VAR1: 60/61-62 °C primestar/RF enzyme
40/45-72°C I5
16:00
Result: The 61-62 °C primestar strip is bright and in the correct position;
Banded at 60-62°C but darker.
PCR reconnects COX1, COX2, ATP8, ATP9

18:00
COX1, COX2, ATP8, ATP9 transformation, plating culture.
Colony PCR: 6 parts each of ATP6, COB and COX3
Nucleic acid electrophoresis verification: COX3 strips are bright and in the correct position, and the rest have no obvious bands.
19:00
PCR: VAR1 primestar 200ul 61-72°C
Nucleic acid electrophoresis verification: the strips are brighter and have a miscellaneous band.
Glue recovery, measured concentration: 55.39
20:30
Activate two ATP6, COB and COX3 (2, 6)
Each of the two monoclonal antibodies was activated.
Conversion results: ATP8, COX1, COX2 grew colonies, and ATP9 didn't.

2018.8.1
9:00
Bacteria: COX3, ATP6, COB plasmid.
PCR: ATP9 200ul I5
Nucleic acid electrophoresis verification: the bands are bright and no bands.
Glue recovery, measured concentration: 127.
10:30
PCR: COX3, ATP6, COB 10ul 55-72 ° C I5 30s 25×.
Nucleic acid electrophoresis verification: COX3 is in the correct position.
Activated ATP8, COX1, COX2 to 2ml EP tubes linked yesterday.
14:00
Linked ATP9, VAR1
Transformed DH5α and plated.
Colony PCR: ATP8, COX1, COX2 T5 Super mix
Nucleic acid electrophoresis verification: ATP6 position is small and COB position is correct.
Activate ATP8, COX1, COX2 4, 6.

2018.8.2
9:00
Plasmids: ATP8, COX1, COX2, 4, 6.

PCR: ATP8, COX1, COX2 10ul I5
12:15
DMT digestion PSB1C3
picked bacteria and activated ATP9 VAR1 each 6 .
15:00
Result: ATP8 was correct.
COX1 and COX2 were rePCR.
Nucleic acid electrophoresis verification: The COX2-4 strip was correct.
15:15
Gibson is diluted with COB 5 times, 50 ° C, 1 h, 2 ul;
Vazyme was diluted 2 times with COB, 32 ° C, 40 min, 1.12 ul.
18:30
COB coated, cultured at 37 °C.
19:00
Colony PCR Verification: ATP9 and VAR1, T5 Super mix 20ul
ATP9 had a correct strip and VAR1 didn't.
Take two activations of ATP9 and VAR1.
The activated COX1-1 (with red and white colonies) transformed by 7.31 was activated.

2018.8.3
9:30
Colony PCR: 4 COX1 red colonies and 6 white colonies.
Nucleic acid electrophoresis test: White-6 has a band and the position is incorrect.
3 and 6 were each transformed into 5 ml and cultured at 37 °C.
Plasmids: ATP9, VAR1, concentration, low concentration.
PCR: ATP9, VAR1 I5 10ul
Nucleic acid electrophoresis test: ATP9 had a band and VAR1 didn't.

16:30
PCR VAR1 60-72°C I5
Nucleic acid electrophoresis test: the band was dark.
Fusion PCR: ATP6
Nucleic acid electrophoresis test: the band was dark and not pure.
extracted the plasmid：COX1 concentration

3	35.00
6	81.08

19：00
PCR：COX1 I5 10ul
Nucleic acid electrophoresis verification: 6 strips were positioned correctly and brightly. (Segment contrast was darker?)
Colony PCR: COB Super mix 20ul
Nucleic acid electrophoresis verification: no bands.

2018.8.4
9:30
Activated COB 4 groups, COX1.
10:30
PCR: VAR1 10ul primestar/I5 61-72°C
Primestar 61-62°C
Nucleic acid electrophoresis verification: no bands.
16:30
Plasmid: COB
PCR: COB I5 10ul
Nucleic acid electrophoresis test: band was dark (suspicious)
Bacteria: COX1
19:30
Pick monoclonal culture ATP6, COB
Activate ATP9, COB V-2

2018.8.5
8:30
Colony PCR: 9 COB V, COB G, ATP6 6 10ul Super
Nucleic acid electrophoresis test: ATP6, COB G, COB V-1 were not correct.
ATP6U, ATP6D was examined by nucleic acid electrophoresis: the bands were bright and in the correct position.
Glue recovery, measured concentration: 103.1, 107.3.
Activation: ATP 6-6, COB V-1, COB V-6, COB G-8.
Fusion PCR: ATP6 (calculated according to 0.04 and 0.02 respectively)
Step1 10ul I5
Step2 20ul I5 nucleic acid electrophoresis verification The strip is bright and in the correct position.
200ul I5
14:00
Glue recovery, measured concentration: 70.78, 28.73
Link PCR: ATP6 vazyme 55 ° C for 25 s.
Plasmid: ATP6-6, COB V-1, COB V-8, COB G-8
19:00
Transformed, plated.
Nucleic acid electrophoresis test: no bands.
2018.8.6
Link PCR: ATP6 primestar 20ul 55-72°C
Nucleic acid electrophoresis test: ATP6-6, COB V-1 bands were bright and in position; COB V-8, COB G-8 have no bands.
PCR VAR1 primestar 61-62°C 20ul
Nucleic acid electrophoresis test: the strip is bright and in the correct position.