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Overview (16.04)

1. plasmid extraction

- ① plexA (\rightarrow Fis2cyto) (DBD)
- ② pB42AD (\rightarrow HopA01) (AD)
- ③ plexA - EFRcyto (g1670)

Selection marker = Carb

2. transformation

- p909 HopA01 - FLAG
- 910 HopA01 (C \rightarrow S) - FLAG
- 911 HopA01 - HA
- 912. ... (C \rightarrow S) ...

- p1164 pBD-pDEX
- p1627 pB42AD - Fis2 cyto.
- p1954 plexA - EFR cyto.

Streak

g1663

g1667

g1678

from glycerol stock

3. PCR

EcoRI - HopA01 - XbaI
(C \rightarrow S)

4.

check plexA pB42AD plexA-EFRcyto

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Plasmid DNA extraction (omega 30-Tek).

1. Grow 1.5ml culture overnight in 10-20ml culture tube.
 2. Centrifuge at max speed for 2'. Remove SV.
 3. Add 250µl Solution I (with RNase A). Vortex to mix thoroughly. Transfer suspension in a new 1.5ml microcentrifuge tube.
 4. Add 250µl Solution II. Invert & gently rotate the tube several times until a flocculent white precipitation forms. Centrifuge at max speed for 10'. Compact white pellet will form. Promptly proceed to next step.
 5. Insert HiBind DNA mini column into a 2ml Collect Tube.
 6. Transfer cleared SV by carefully aspirating it into column. Centrifuge at max speed for 1'. Discard filtrate & reuse collection tube.
 7. Add 50µl TBC Buffer diluted in 60% isopropanol. Max speed 1'. Discard filtrate.
 8. Add 700µl DNA wash buffer diluted in 100% ethanol. Centrifuge at max speed 30'. Discard filtrate. Repeat once more.
 9. Centrifuge column at max speed 2' to dry column. Transfer column to eppi.
 10. Add 30-loopful elution buffer. Wait 1'. Centrifuge at max speed 1'.
 11. Store at -20 °C
- Tubes :** plexA pB42AD pLexA-EFR
- X 2 tubes
- 1 → total 6ml culture per tube

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CONCENTRATIONS

- 1 plexA-EFR - 53.6 ng/μL
 2 plexA-EFe - 41.6 ng/μL
 3 pB42AD - 730.3 ng/μL
 10^4 pB42AD - 80.2 ng/μL
 5 plexA - 40.5 ng/μL
 6 plexA - 50.0 ng/μL

PCR protocol

50 μl System

- 32 μl ddH₂O
- 10 μl 5x HF Green buffer
- 5 μl dNTPs
- 1 μl Fwd primer (10 μM)
- 1 μl Rev primer (10 μM)
- 1 μl X7 polymerase
- \sim 10 ng template.

p911:	181.1	0.15
p912	160.9	0.15
p910	150.5	0.15
p909	64.4	0.3

PCR strip: 1 - p909
 2 - p916
 3 - p911
 4 - p912

X2 DNA for transformation

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PCR programme:

- 95°C 3'
- 95°C 30"
- 56°C 30"
- 72°C 1'
- 72°C 2'
- 10°C 30"

Volume: 60 µl

Transformation of plasmids into E. Coli:

Concentrations

λ 164	2.2 ng/µl
λ 1627	19.7 ng/µl
λ 1954	13.6 ng/µl

5
1
1

→ DNA for transformation
some plasmid as used for PCR

Protocol:

- take eppis with E. coli from -80°C & keep on ice until thawed
- Use 25 µl \rightarrow for pure plasmid transformation. 10 µl w/o vector for λ 164 use 50 µl except for ligation product
- DNA volume as noted next to concentration
- Leave cells on ice for ~20'
- Heat shock at 42°C for 45"
- Put back on ice for 1-5', add 5 µl LB, incubate 30' (37°C)
- Plate on selection media.

* λ 909 - λ 912 \rightarrow Spectinomycin (Spec)

* λ 164

\rightarrow Kanamycin (Kan)

* λ 1627, λ 1954 \rightarrow Carbenicillin (Carb)

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PCR results

- 5 → p₉₁₀ showed correct band but weak (non-spec bands also present)
 → no extraction (will extract from unpurified plasmids from E. coli)

E. coli plates

- 10 → all transformations successful
 (1,000 x dilution abs)

Liquid culture protocol

- 15 → 4 mL LB
 → 1000 x dilution of stock antibiotic
 ↳ stock conc. ab.
 carb: 100 µg/mL
 kan: 50 µg/mL
 spec: 100 µg/mL

- 20 → pick colony
 → incubate overnight in shaking incubator (37°C)

rd Y2H things received

- 25 → yeast strain OGY216 (solid culture)
 → plasmid pACT2 - PTAP (amp 50 µg/mL) (carb) → 42.6 ng/µL
 → plasmid pLexA - TsG101 (amp 100 µg/mL) (carb) → 28.3 ng/µL
 30 → protocol

PLATING OGY216

- 35 → Stab sample resuspended in ~20 µL water
 → 5-10 µL streaked on YAPD plates
 → incubate at 28°C

pACT2 empty vector in E. coli in 37°C

Transformation into E. coli (protocol pg 4)

- 40 → pACT2 - PTAP
 → pLexA - TsG101
 → pGBKT7 → EV
 45 → pADT2 - EV (amp)

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PLATES Poured (carb ampicillin)

- ca. 15mL agar 1 or 2% plate, 1 cm. 5 mL for small spots
 → 1000x antibiotic dilution.
 → plates in fridge.

SEQUENCING (microarray)

- DNA at concentration of 40-100 μ g/ μ L
 → 12 μ L total
 → pLexA, p^{B42AD}, pLexA-EFR sent for overnight sequencing
 (to certain ADH1 primers).

15

18/4/23

Plasmid extraction (protocol pg 2)ng/ μ L

20	Final concentration: (in 70 μ L)	Picomolar	
		1	2
21	1627	312.3	349.2
22	1663	66.4	104.5
23	1667	89.7	61.2
24	1638	66.8	67.5
25	1951	73.7	92.1
26	909	86.7	118.5
27	910	29.0	81.3
28	911	115.7	82.6
29	912	107.8	53.7
30	1164	14.8	14.2

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17/4 - 18/4

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*Continued from Page***PLASMID EXTRACTION** (OMEGA Mini Kit)

- PGK KT7 (2 replica)
- pACT2 PTAP (2 replica)
- pACT2
- plexA TSG101
- pG4D TT

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PCR of HopA1 & FLS2

→ protocol from page 3

→ Run a gel (~~Agarose/Amber~~)

↳ all PCRs were successful!

→ Cut the bands out of the gel & gel extracted

following protocol from Omega - BiTek kit

→ 600 µL of buffer XP2 at beginning

→ 10 min at 70°C shaker at 60°C

→ Did 2 washes with buffer SPW + EtOH

→ Eluted in 60 µL MQ water

→ Nanodrop

	C	260/280	260/230
C>S 1	114 mg / µL	1.87	0.79
C>S 2	75.2 mg / µL	1.88	1.51
Hop 1	129.9 mg / µL	1.92	1.54
Hop 2	116.9 mg / µL	1.93	0.96
FLS2	119.8 mg / µL	1.89	2.17

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2023.4.15

can be used as positive control with plexA-Tsg101 in plexA Y2H system!

Sequencing alignment

\rightarrow XbaI cutting site is still there
 \rightarrow need to design new primer to amplify FLSr reverse

• pACT2-PTAP

C2LP1954 - plexA-EFRcyto correct

for insert into this vector
 (primer iDEC4 has stop codon)

g1678 plexA-EFRKD correct

\Downarrow
 without IJM and tail

g1663 plexA-FLS2KD X frame shift!

(g1670 plexA-EFRcyto X also has frame shift!)

g1667 plexA-BAK1KD partially correct (without stop codon...)

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① Digestion + Ligation

→ plexA + FLS2 (stop codon)
 → pB92AD + HopA01

New PCR

② PACT2-PTAP ← FLS2cyto (without stop)
 PGK-T7 ← HopA01

③ PACT2 ← FLS2cyto (with STOP)

Digestion:

30 μl system

Enzyme A 2.5 μl

B 2.5 μl

10x Buffer 5 μl

Template 2 μg

dH₂O 50% to 20 μl.

EcoRI

3.1 50%

Cutsmart 100%

BamHI

3.1 100%

Cutsmart 100%

{ XbaI

3.1 100%

Cutsmart 100%

{ SalI

NEB 3.1 100%

{ pFLS2cyto BamHI XbaI

PCR-HopA01

EcoRI XbaI

plexA

BamHI XbaI

- USE NEB 3.1 for

all

3.1

pB92AD

EcoRI

XbaI

Cutsmart / 3.1

3.1

ACT2/ACT2-PTAP

BamHI

XbaI

Cutsmart / 3.1

pGBKT7

EcoRI

SalI

3.1

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Digestions

PCR - FLS2 - cyto, PCR - HrpA01, plect

- BamHI & XbaI
- EcoRI & XbaI
- BamHI & XbaI

PCR tubes

1 - FLS2 cyto (17 μL DNA)	- 24 μL water	} cutsmart
2 - HrpA01 (19.54 μL DNA)	- 23.5 μL water	
3 - pGexA (40 μL DNA)	- 4 μL water	} Buffer
4 - pB424P (15.35 μL DNA)	- 25.65 μL water	
5 - pACT2 (35.21 μL DNA)	- 5.8 μL water	} 1 μL DNA / 22.5 μL water
6 - pACT2-PFAP (24.5 μL DNA)	- 16.5 μL water	
7 - pGBKT (4.86 μL DNA)	- 36.2 μL water	} NEB 3.1 mmath Buffer

(gel extraction 3.1 mmath, 1.5% agarose, 1.5V, 45 min, 4 °C) - A. 4.1g

A. 4.1g (3.1 mmath, 1.5% agarose, 1.5V, 45 min, 4 °C) - 1.5V, 4 °C

A. 4.1g (3.1 mmath, 1.5% agarose, 1.5V, 45 min, 4 °C) - 1.5V, 4 °C

2.8 (3.1 mmath, 1.5% agarose, 1.5V, 45 min, 4 °C) - 1.5V, 4 °C

Invert gel after 40 min at 1.5V, 4 °C

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Ligation

1. Combine:

→ Vector DNA (~100ng)

→ Insert DNA

→ Ligation Buffer 10x (1μL/10μL reaction) + 1μL Ligase/10μL system

2. Incubate at room temp for 2 h. or 16°C overnight.

Vector and insert sizes

- pLEX A (10Kbp) 14.7ng/μL
- pB424D (4.6Kbp 6.4 Kbp) 25.0 ng/μL
- pACT2 (8Kbp) 23.2 ng/μL
- pACT2-PTAP (8Kbp) 21.8 ng/μL
- pGBT7 (3.6Kbp) 7.3 Kbp 53.1 ng/μL
- FL52 (1Kbp) 36.3 nJ/μL
- HopA01 (7.5Kbp) 27.1 ng/μL

Amounts

- | | +20 |
|---|-----|
| pLEX A - FL52 → 100ng pLEX A (7μL), 30ng FL52 (1μL) | 0 |
| pB424D - HopA01 → 100ng pB424D (4μL), 85ng HopA01 (3μL) | 1 |
| pACT2 - FL52 → 100ng pACT2 (4.5μL), 37.5ng FL52 (1.5μL) | 2 |
| pGBT7 - HopA01 → 100ng pGBT7 (2μL), 67.6ng HopA01 (2.5μL) | 3.5 |
| (Digestes vector as negative control) | |

PCR step:

- 1- pLEX A negative control
- 2- pLEX A - FL52
- 3- pB424D negative control
- 4- pB424D - HopA01
- 5- pACT2 negative control
- 6- pACT2 - FL52
- 7- pGBT7 negative control
- 8- pGBT7 - HopA01

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E. coli TRANSFORMATION OF CLONED PRODUCTS

- pLexA → ampicillin
5 → pBAD → ampicillin
→ pACT2 → ampicillin
→ pGBT9 → chloramphenicol

10 → 5 μL ligation product added for transformation by heat shock

15

20

25

30

35

40

45

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- 5 • Redo the PCR, Digestion, Ligation.

opti-
mization

1. PCR clean-up
 2. BamH1-HF... new version of enzyme → all compatible with Cutsmart
 3. new enzyme.
 4. Ligation 16°C overnight

5.7

- 25 1. Colony PCR. (25 μL)

- 5 constructs X 9 colonies each

• controls : EV

ζ ζ ζ as f primer
 all others use 215 f. 216 R

- 30 2. plasmid extraction

• PACT2

• PGBT7 - PS3
 • PGADT7 - AGT }

35 PC for GAL4 Y2H

• pACT2-FLS2 cyto

• pB42AD- HapA01 cys

• PGBT7- HapA01 cys

• PACT2- FLS2 cyto

• PACT2- FLS2 cyto- PTAP
 (w/o stop)

35 FLS2cyto: 1000 bp

HapA01: 1500 bp

5 533
 5 5
 5 5

- 40 3. LB

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1 reaction

16 μ L dH ₂ O	- X
5 μ L HF Green Buffer	X
2.5 μ L dNTPs	X
0.5 μ L FW primer (10 μ M)	
0.5 μ L REV primer (10 μ M)	X
0.5 μ L Polymerase (x7)	

52 reactions (45 + 15C + 2 extra)

832 μ L
260 μ L
130 μ L
26 μ L \rightarrow 5.5 μ L (30S FW)
26 μ L \rightarrow 20.5 μ L (21S FW)
26 μ L \rightarrow 5.5 μ L
26 μ L \rightarrow 20.5 μ L (27.5 μ L)

→ Prepare MM with all but FW primers, take away 11 reactions for pBG2AD-HspA01 C→S which needs a diff FW primer (30S) and use 21S for rest

Primers

FW 21S RV 216 \rightarrow pLacA-FLS2 cyto
 \rightarrow pGBKT7-HspA01 C→S
 \rightarrow pACT2-FLS2 cyto
 \rightarrow pACT2-FLS cyto-PTAP

FW 30S RV 216
 \rightarrow pBG2AD-HspA01 C→S

Julien Kion Ale C
MM1 pLacA-FLS2 cyto
MM2 pBG2AD-HspA01
MM1 pGBKT7-HspA01
MM1 pACT2-FLS2 cyto
MM1 -II- PTAP
* 5 are switched
25 mL

→ MM Prep

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Concentrations :

5	PGBKT7-053	:	470.3	366.7
	PGBT7-AgF	:	368.7	163.9
	pAct2	:		44.1

10	PGBKT7-053	:	345.7	
	PGBT7-AgF	:	179.5	
	pAct2	:	43.2	

Loading of PCR colony:

original sample then control

Lanes 1-3 (Agt2-G-298)

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Plasmid extraction

- pACT2-FLS2cyto X
- pB42AD-1TOPK01-3 ✓
- pGBKT7-HopA01 ✓
- pB42AD-1TOPK01-1 ✓

collected 3ml of culture

followed E.Z.N.A. Plasmid DNA Mini Kit 1 protocol → sent for sequencing

redo gel for -plexA-FLS2cyto (24 colonies) - pGBKT7-HopA01 (12 colonies)

- pAct2-FLS2cyto - PTAP - pAct2-FLS2cyto (12 colonies)
(24 colonies)

start liquid culture for ~~plexA-FLS2cyto~~

g.5

Extraction of the colonies

- 87 plexA-FLS2cyto-12
- 88 pAct2-FLS2cyto-PTAP
- 89 pGBKT7-HopA01-2
- 90 pAct2-FLS2cyto-9

elution nptl come /mg ml⁻¹
58.1
59.0 ±

327.3 x 5
107.5 x 2

12 μL

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• yeast

- for forward Y₂H

- EGR48 (LacZ reporter) — liquid culture (-u)

- AP4-fRP795 (FP reporter) — streak on plate (-u)

- for reverse Y₂H

OVY₂16 — liquid culture (YPD)

→ 2 of each plate (colonies)

+ 1 liquid culture of OVY₂16
from the author

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• plasmids

• PB42AD-HopA01 ✓

• pGBKT7-HopA01 ✓

• plexA-FLS2cyto (might without STOP?)

• PACT2-FLS2cyto (w/o STOP, but fine)

• PACT2-FLS2cyto-PTAP (w/o STOP, need to redo)

• prepare for the transformation

T_{RP} His - PB42AD-HopA01 + plexA-FLS2cyto

(plexA system)

- EGY48 (science)
- lacZ

(- - - - + -- EFRcyto)

- AP4-FRP-795 (with FP)

PC: (Leu) (His)

- PACT2-PTAP + plexA(T202)PL-T5610

(chisLeu, trp)

- BAK1-BIR2 (from Songyuan)

- T_{RP} Leu - plexA-VP16 (- His)

NC:

(- PB42AD + plexA)

YPS

- PB42AD-HopA01 + plexA

- PB42AD + plexA-FLS2

(- - - - - EFR)

X187

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Stock solution:

to make 100 mL
medium

50 mL

2X

Agar
(100 mL)

40 g/L

4 g/
100 mL20% Glucose
(20 mL)20 g/
100 mL

10 mL

10X

SD-d dropout (-Ura/-Leu/-Trp)
His 14.6 g/L
(-Ura/-Leu/-Trp) 1.46 g/
100 mL 10 mL

YNB

160 mL 1.34 g/
1.25 60 mL2.23 g/
100 mL

30 mL

10%

Raffinose + Galactose

20%

20 g Gal + 10 g Raf

/100 mL

LIQUID CULTURE EGY48 A 24-F2P-395

→ Ura - uracil medium used
→ 1 mL medium + 1 colony
→ 28°C

100 mL medium

→ 50 mL agar (or water for liquid medium)
→ 30 mL YNB
→ 10 mL 20% Glucose
→ 10 mL 10x SD dropout

29.1

- LW CM plates
- LW plates.

- Chitin Stock solution

Draw #8 → APS box
Colloidal chitin polysaccharide 10 mg/mL
Zentel Biopolymer uses

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YEAST TRANSFORMATION

→ Mix:

PEG 3350 (50% w/v)

7

5

10

Lithium acetate 1M

240 μ L ✓36 μ L ✓

(Positive) ss carrier DNA (2ug/ml)

50 μ L

Plasmid + water

34 μ L ✓

Total volume

360 μ L

→ 500 ng DNA per plasmid added, ss carrier DNA boiled at 95°C then cooled in ice box.

→ centrifuge yeast cells (3000g 5 min acceleration g) discard supernatant

→ warm cells (resuspended in water v) and centrifuge (same settings)

→ resuspend pellet in 1mL 0.1 M Lithium acetate.

→ transfer to 1.5 mL tube

 $900 \mu\text{L H}_2\text{O} + 100 \mu\text{L } 0.1 \text{ M LiAc}$

→ Centrifuge (max speed, 1 min)

→ resuspend in 150 μ L water. (less is ok)→ add 10 μ L yeast cells to reaction mixture, vortex and incubate for 10 min (28°C)

→ heat shock: 1 hour at 42°C

→ centrifuge (1 min max speed) resuspend in 500 μ L water→ plate 60 μ L

→ incubate at 28°C. 2+ days.

Total culture 370 μ L

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PCR cleanup for FLS2, FLS2 no stop.

Digoxigenin

→ DNA	10 μ L
10x NEB restriction	5 μ L
BAMHI	1 μ L
XbaI	1 μ L
water	33 μ L

→ incubate at 37°C for 1 hour

→ Come after cleanup

FLS2 no stop 19.93 μ g μ L $^{-1}$ → Ok, only 19.93 μ g were digested
 FLS2 19.04

Ligation

pEX A with STOP

PTAP

pACT2' Y no STOP

pACT2 with STOP

Ligation - λ EOP system.

→ 100 ng backbone

→ 1ms/bb 4:1

→ Buffer 2 μ L

→ 1.5 μ L ligase

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$\rho\text{ACT2 PTAP (3 kbp)} \rightarrow 20 \text{ fmol}$
 $\rho\text{ACT2 (8 kbp)} \rightarrow 20 \text{ fmol}$
 $\text{plexA (10 kbp)} \rightarrow 16 \text{ fmol}$
 $\text{FLS(1 kb)} 13,04 / 14.93 \text{ mg } \mu\text{L}^{-1}$
 $\downarrow \quad \downarrow$
 $20 \text{ fmol } \mu\text{L}^{-1} 24 \text{ fmol } \mu\text{L}^{-1}$

Reactions $\rho\text{ACT2 + with stop}$ backbone $9 \mu\text{L}$ ✓insert $4 \mu\text{L}$ ✓H₂O $8.5 \mu\text{L}$ ✓ lexA + with STOP backbone $11 \mu\text{L}$ ✓insert $3 \mu\text{L}$ ✓H₂O $2.5 \mu\text{L}$ ✓ $\rho\text{ACT2 PTAP + no STOP}$ backbone $6 \mu\text{L}$ ✓insert $8.5 \mu\text{L}$ ✓H₂O $7 \mu\text{L}$ ✓ $\rightarrow \text{All have } 2 \mu\text{L buffer & } 1.5 \mu\text{L }$

Ligase

 $\rightarrow 3 \text{ h. at } 16^\circ\text{C}$

Lyophilize

July 17, 1988 S2 S257-S258

July 19, 1988 P2 S257-S258

July 19, 1988 P2 S257-S258

July 19, 1988 Q2 S257-S258

July 19, 1988 R2 S257-S258

July 19, 1988 T2 S257-S258

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Colonies PCR of FLS2 transformation

- 5 → 14 colonies for each (pACT-FLS2*, pACTA-FLS2*, pACT-FLS2-PTAP*)
 6 positive control: respective purified plasmids (not tap)
 6 25 μL system (MM for 50 reactions prepared).

10 → Colony PCR successful
 11 → 3 colonies of each transformation inoculated

15 → Collected concentrated E. coli PCR plated
 16 → Centrifuged, 1/2 supernatant removed, resuspended.

18.5.

→ All grow

↓
Same color change

pACT2-FLS2 22 84.7 mg

pACT2-FLS2 25 25.9 mg/μL

pACT2-FLS2 26 24.9 mg/μL

pACT2-FLS2 PTAP 70.2 mg/μL → Fuchs this one

pACT2-FLS2 PTAP 16 25.1 mg/μL

pACT2-FLS2 PTAP 27 26.6 mg/μL

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PLASMID EXTRACTION plexA-FLS2^f

5 4 colony 2 → 57.4 µg/µL
4 colony 3 → 66.0 µg/µL
4 colony 4 → 52.5 µg/µL

10 plexA - EV Liquid culture set up

250 µL

21.5.23

make media

?
make 20 mg/ml
stock solution in
DMSO

- solid:

SD-Galactose/raffinose - UWH with 80 µg/ml
(50 ml) X-Gal

- liquid

• SD-Glucose - UWH (50 ml)

• SD-Galactose/raffinose - UWH (50 ml)

Yeast culture

Row 1 Pink

APG 1-4, NC2 1-4, NC1 1-4

~~PL2A1, PL2B1, PL2C1, pBAD42A1, pBAD42B1, pBAD42C1, plexA-FLS2~~

(All APG)

Row 2 white

NC2 1-4, EG398 1-4, NC1 1-4 (All EG398)

Row 3 - white

Positive control (3Y)
APG

APG	exp	NC2	NC1
EG398	NC2	exp	NC1
APG	PC		

OMES ~~strains~~ named as
the strain we
experiment

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Please Supply Vector plasmid extraction

Dilute 1: 97.9 ng/ μ L
Dilute 2: 108.7 ng/ μ L

→ PLATING YEAST ON X-GAL PLATES

↳ Plate 1: 10 μ L per culture

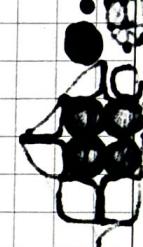
Row

1	AP4 - HlapA1 + FCS2
2	None AP4 - NC1
3	None AP4 - NC2
4	EGY48 - HlapA1 + P2S2
5	EGY48 - NC1
6	EGY48 - NC2

23.2.21

23.2.18

↳ Plate 2, same layout, but 5 μ L per culture plated



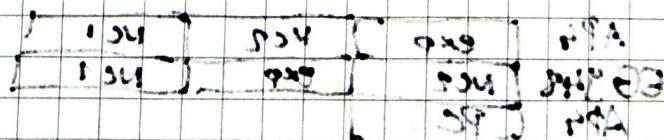
→ Culture prepared for FACS.

↳ 800 μ L medium (Catalase + Raffinose)
9 10 μ L colony

(23.2.18) → 15V, 2-1 53V, 2-1 49L
→ 22.2.18 15V, 2-1 53V, 2-1 49L
(23.2.18) → 15V, 2-1 53V, 2-1 49L
→ 22.2.18 15V, 2-1 53V, 2-1 49L
(23.2.18) → 15V, 2-1 53V, 2-1 49L
→ 22.2.18 15V, 2-1 53V, 2-1 49L
→ 22.2.18 15V, 2-1 53V, 2-1 49L

↳ 1000 μ L medium

→ 22.2.18 15V, 2-1 53V, 2-1 49L



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NATURE	DATE	WITNESS	DATE
	22/5/2023		

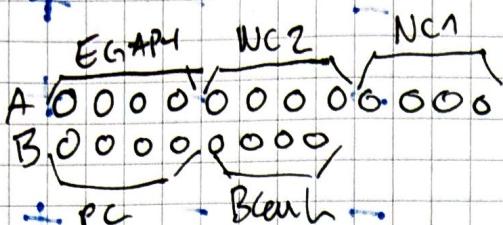
TITLE

PROJECT

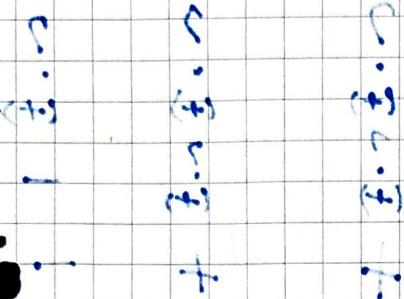
DATE

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TAGS



C → same as A, higher Voltage.



1990 01

1990 01

5

10

15

20

25

30

35

40

45

1990 01

1990 01

1990 01 (1) TgA - FT(A-)

1990 01 (2) E29 - FTX8-9

Continued to Page

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AD prey

DBD bait

lacZ

Ura

HIS

NCl : pACT2-PTAP ✓ + pGBT7

- - +

Nc2 : pACT2-FS2-PTAP
cyto ✓ + pGBT7

- - +

Nc3 : pACT2-PTAP ✓ + pGBT7-HopAo1 ✓

- - +

EG : pACT2-FS2-PTAP
cyto ✓ + pGBT7-HopAo1

? ? ?

Ncf : pACT2-FS2
cyto ✓ + pGBT7 - HopAo1 ✓

(+) (+) (+)

PL : pGADT7-AgT + pGBT7-p53

+ + -

CONCENTRATION

pGADT7-AgT (1) 140.8 ng/mL

pGBT7-p53 (1) 343.3 ng/mL

pACT2 (PTAP) → Leucine

pGBT7 → Tryptophan

pGADT7 → Leucine

SIGNATURE

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DATE

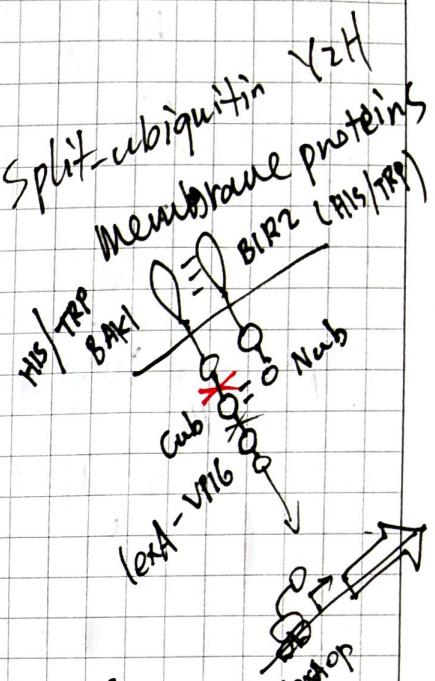
TITLE

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- start overnight culture for RDY2H (25.5) ^{did on}
(HIS, TRP)
- repeat the lexA Y2H
 - miniprep for PC plasmids \Rightarrow
 - make plates (-HIS, -TRP, -URA)
 - transformation (tomorrow)

Min'prep

our positive controls

\rightarrow His BIR2 - mub \rightarrow both 2
 \rightarrow Trp BIR2 - mub

ATCC 6 366.2
 10 218.5
 3 315.0

KD 1 181.5
 2 210.1

Bik 3 273.0
 4 256.0

SKOR 1 71.2
 7 232.6

His BIR3 - Nub 1 223.6
 2 217.7

Trp BIR3 - Nub 1 270.8
 2 296.4

- U, W, H, L + L
 \downarrow
 0.38
 880 mg/L
 $20 \times 7.6 \text{ g/L}$

380 mg/50ml
~~Stock Solution~~

\rightarrow 50 mL -Leu. -Trp

5 Glu
 5 drop -Leu -Trp
 15 YPD

25 Water
 \rightarrow 50 mL

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15 ml

30

20

DATE

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FLS2

NC1: plexA ✓

pB42AD ✓

NC2: plexA-FLS2 ✓

pB42AD ~~HopA01~~ ✓

NC3: plexA ✓

pB42AD-HopA01 ✓

EG: plexA-FLS2 ✓

pB42AD-HopA01 ✓

PG1: ~~BAK-Luc~~ ✓TRP ✓
BIR-Nub

PG2: p10 LexA-VPI6 ✓

p09 ✓

EFR

NC4 plexA-EFR ✓

pB42AD ✓

EG: plexA-EFR ✓

pB42AD-HopA01 ✓

34 pm 10
 5.008 2 0.078
 2.812 01
 0.218 8

-Trp, -leu, -ura + X-gal 100 μ → some induction? ✓
 1.012 8

0.872 8 4.02
 0.421 0

-Trp, -leu, ~~-ura~~ - IaaCip ✓

5.14 1 8.032
 4.222 7 F

ura 2
 leu 2
 ura 2

8.032 1 6.01-8.032 2.02
 5.412 7

ura 2
 leu 2
 ura 2

8.032 1 6.01-8.032 2.02
 5.412 7

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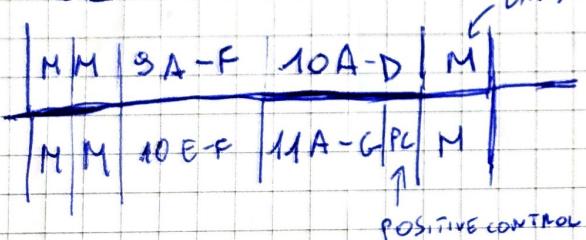
PLATE ASSAY PREPARATION

- 5 → ~~500 μL~~ 500 μ L culture taken into 1.5 mL tube
 → Centrifuge (3 min max speed)
 → Resuspend in 1 mL water
 10 → Normalize OD (goal \rightarrow 0.2 OD₆₆₀)
 → Serial dilution
 15 (↳ final 1:1,000 in jumps of 1:10)
 → Plate 5 μ L per spot dilution (sample)

02-06

BACKUP

GEL LOADING ORDER:



9 AVRPTO

10 AVRPTO GA \rightarrow MUTATION

11 AVRPTOB

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08.06.23

- 5 • miniprep : 3 constructs x 2 replicates (colonies)
- Aur Pto
 - Aur Pto - G2A (mutation)
 - Aur Pto B

Concentrations :

• Aur Pto - 5	72.4
- 6	68.3
• Aur Pto - G2A - 5	55.1
- 6	55.9
• Aur Pto B - 5	50.1
- 6	24.2

05.06.23

MINIprep

260/280

260/230

• LVC 1	234.0 ng/mL	1.87	2.26
• LVC 2	316.6 ng/mL	1.84	1.87
• SNRK2	192.1 ng/mL	1.86	2.31
• SNR6	284.6 ng/mL	1.84	1.98
• SKO 1	317.8 ng/mL	1.86	2.31
• SKO 2	271.6 ng/mL	1.82	1.89
• SKO 5	262.5 ng/mL	1.85	2.33
• SKO 6	300.0 ng/mL	1.87	2.31

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6.6.23

Generate mutations on AvrPto and AvrPtoB

① 8, 11 (1473 bp)

② 10, 9 (260 bp)

③ 12, 16 (13) - 173 bp

④ 15, 14 393 bp (G2A)

⑤ 12, 18 (13) - 302 bp

⑥ 17, 14 264 bp (G2A)

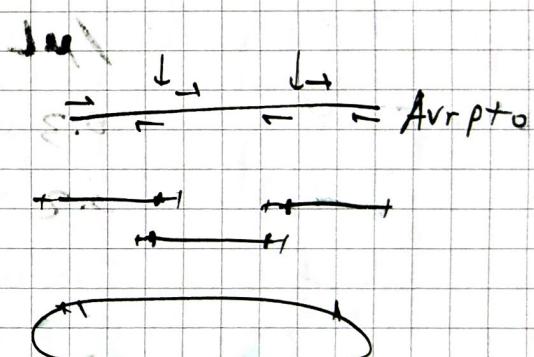
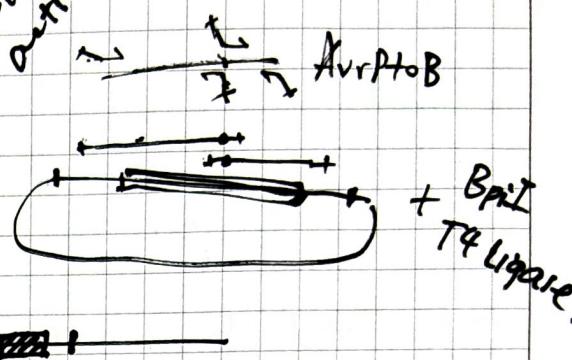
⑦ 12, 16 (13) (173 bp)

⑧ 15, 18 (153 bp) } AvrPto S46P, Y89D

⑨ 17, 14 (264 bp) (G2A).

AvrPtoB F479A

Kill EG-activity

PCR : 50μl System

- 32 μl ddH₂O
- 10 μl 5x HF Green buffer
- 7 μl dNTPs
- 0.2 μl FW (100 μM)
- 0.2 μl RV (100 μM)
- 1 μl 7x polymerase
- 1 μl template (Pto: 1.46 μl, PtoB: 2 μl)

MM AvrPtoB : 2 samples

MM AvrPto : 7 samples

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PCR strip498 μ L MM
P10 B

1 2
 Pimers Pimers }
 3 4 5 6 7 8 9
 11, 12 13, 14 15, 16 17, 18

498 μ L MM
P101 μ L each

2) Run samples on agarose 1% gel

3) Perform gel extraction with
omega Biotech kit

4) Calculation of molar ratios:

(1. 78 fmol μ L ~~at 2 μ L~~

2. 553.2

0.3 μ L

3. 991.2

0.2 μ L

4. 535.2

0.3 μ L

5. 401.9

0.35 μ L

6. 393.0

0.4 μ L

7. 326.4

0.5 μ L

8. 528.6

0.35 μ L

9. 323.6

0.5 μ Lbackbone 1 μ L 43 fmol

129 fmol

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Goldengate reaction (10 μL).

F479A S46P Y89D S46P
Y89D

5	backbone	1 μL				
10	insert	X μL	2.3	0.5	0.75	1.35
15	10X T4 ligase buffer	1 μL				
20	T4 ligase	0.5 μL				
25	BpiI (Esp3I)	0.5 μL				
	ddH ₂ O	to 10 μL	4.7	6.5	6.25	5.65

07-06-23

TRANSFORMATION OF MUTATED AurPto AND AurPcoB IN E.COLI

- EPPIS WITH E.COLI FROM -80°C, KEPT ON ICE UNTIL THAW
- USE 5ML per transformation (SINCE THEY ARE THE RESULT OF GOLDEN GATE LIGATION) AND LEAVE CELLS ON ICE FOR 15 min.
- HEAT SHOCK AT 42°C FOR 45 seconds.
- BACK ON ICE FOR 1-5 MINUTES, ADD 500 μL OF LB MEDIA
- INCUBATE 30 MINUTES AT 37°C
- PLATE 100 μL

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BOOK

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AvrPto - BAK1 / FCS2

PROJECT

4822
JL

DATE

Goldengate

myristylation

Localization test

JL

PM AvrPto - GFP.

PM marker

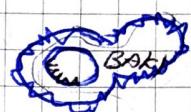
Cyto AvrPto (G2A)

{ Omcherry

am?

10

cyto SsrK2.8



BIK1 (BIK1-FD)

LUC

(CBL-LUC)

LexA-NLS

+ BB07

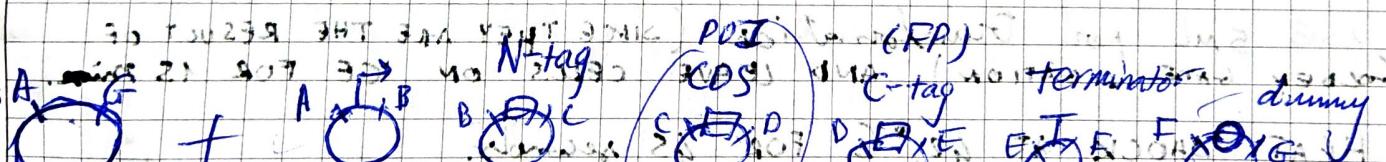
(BL)

ES-00-50

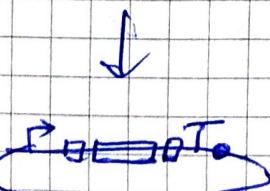
1200.5 4: 8.5% in out 0.0% Cbl-mcherry STAN 200724.97

115W LED no T932, 100% HHS/TPR 21993 •

want



HHS, 9.17, 1, 1002 01A, 2.07.04 2-1 8.07 3-1 10 2003 •

↓
↓ BsaI + T4 ligase

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Pro
ABN-tag
BCP01
(DS)

CD

DE

EF

FG

destination vector pADH1 dummy (BB06) Avrptf1 mGFP TerADH) dummy (BB09)
 LEU 0.7 μl 1 μl 1 μl Avrptf2 (GZA) 1.5 μl 1 μl 1 μl
 AvrptfB 1 μl SsRKZ8.

HIS - pADH1 CBL N12 mCherry dummy TerADH) dummy (BB08) (BB09)

Goldengate mix (10 μl) / (15 μl)

each module > 100 ng

10x T4 ligase buffer	1 μl	1.5
BsaI	0.5 μl	0.75
T4 Ligast	0.5 μl	0.75
ddH ₂ O	to 10 μl	15.

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2023.6.9

- Resend sequencing to Eurofin
- Start overnight culture for localization test
- Goldengate for split-subunit

BB

BC

CD

DE

EF

FG

pOF pADH1 BB06 { ArrPto Nub TerADH1 BB09
 LUC (4) (G2A) S46P Y89D
 S46P, Y89D
 LUC

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Continued from Page		ChmgnC -1 253.9 191.6	PCR 1
1	Avr PTO mGFP 1	1	
2	-11-	2	
3	CBS - mCherry 1		168.0
4	-11-	2	193.0
5	Avr PTOB - mGFP 1		318
6	-11-	2	279.2
7	* Smrk 2.3 GFP 1		263.0
8	-11-	2	181.7
9	Avr PTO G2A mGFP 1	1	211.6
10	-11-	2	170.3
PCR of these			
20	T _{min} = 58° 4 min elongation 1 min		
25		250 mL	
30	Galactose Raffinose - UUHL + Kgal } 66WHL	↑	3 of each 50 mL
35	Mashing yeast → grown in - uuhl media → 500 mL Mg water → 3 min at 17°		
40	→ plated 42H • 2nd try ↳ dilution → NCI NCI NCS NCS FLS EFR		
45			
Continued to Page			
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11.6.2023

5 ~~NEAR~~ mini prep
 LUC mGFP 1,2
 CBL LUC mGFP 4,5 }
 AHA1 mGFP 1,2 }
 BIKI mGFP 1,2 }

Avrpto { S46P 1,2
 Y89D 1,2
 S46P, Y89D 1,2

Avrptob F479A 1,2

15 Localization

20 12 μL samples of 400ng/μL for sequencing

CONCENTRATION mg/mL

25 LVC 1 233.6 •

LVC 2 221.1 •

CBL LVC 4 272.3 •

CBL LVC 5 266.5 •

AHA1 1 284 •

AHA1 2 351 •

BIKI 1 207.8 •

BIKI 2 265.4 •

AVRPTO S46P 1 256.2 •

11 S46P 2 107.7 •

11 Y89D 1 208 •

11 Y89D 2 217.2 •

11 S46P+Y89D 1 224.2 mg/mL •

11 S46P+Y89D 2 201.5 •

AVRPTOB F479A 1 240 •

11 2 235.3 •

976 247 204 204 204 204 204 204

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12.6.23'

→ Colony PCR

1 x vact 50 μ L24 reactions \rightarrow 26 wells32 mL ddH₂O

832 ✓

10 μ L 5x Green buff

260 ✓

5 mL dNTPs

130 ✓

1 μ L FW 12 pm

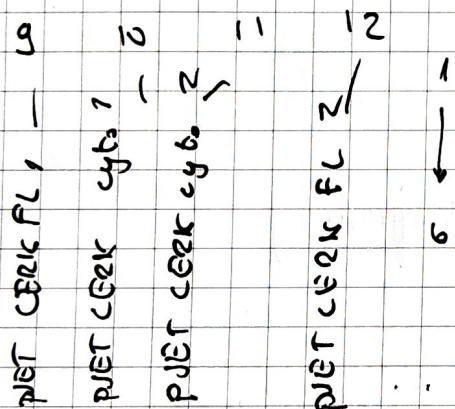
26 ✓✓

1 μ L rT poly

26

→ Not done couldn't find the plates~.

← Close Lsize



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5 1. goldengate assembly to construct Avrpt₀-Nub₀
 (E2A, S46P, Y89D, S46P/Y89D)

	Pro ADH1	BB06	Amp _{To}	Nub	Ter ADH1	BB09
10 Dof LEU						
15 0.7 μl	1 μl	1 μl	G2A 3.3	1 μl	1 μl	1 μl
20 ↓ S-6	8	8	S46P 1.5.3			
			Y89D 1.5.3	8	8	8
			S46P/Y89D 1.5.3			

WT 2 → 4.3

HQ

8/27/23

S. 7 + 3 = 8.7

2. miniprep

PJET-CERK FL

PJET-CERK cyto

⇒ send sequencing

CBL-Luc-Nub 1,2

Luc-Nub 1,2

2. Transformed mutants into competent E. coli

BIK1-Nub 1,2

BIK1 KD-Nub 1,2

MFL FLS2 1,2

MFL HA FLS2 1,2

Gα with Gβ 1,2

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Golden gate

→ pvt AvrPTO 2D, SmRK2.8 & ym UKG61

into am entry vector → Receprie Page 85

(p691) ← bbme

insert (100 ng)

TGC lig buffer (100 ng)

T4 lig

Bpi I (Esp3I)

ddH₂O1
2

AvrPTO

SmRK2.8

ym UKG61

	AvrPTO	SmRK2.8	ym UKG61	
1	2.4	2.8	2.3	✓
2	1	1	1	✓
3	0.5	0.5	0.5	✓
4	0.5	0.5	0.5	✓
5	1 4.6	2 4.2	3 4.7	✓
	10 μL			

→ Diglig program on machine 1 → cycles of 37 & 16 °C

↳ 24 cycles, but com handle as low as 10

→ Started FLY 7D liquid culture → to be transformed

→ streaked Apa + FLP 79S & R270 + FLP 79S

JM - U plate

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PROJECT

1 of 2 is correct

P

DATE

O. aligned pJET-CERK1 FL/cyto sequence

with point mutation
pick other colonies
to check

1. Colony PCR

AvrPto-Nub →

(S) 5 plates x 3 colonies/plate.

S2215, 216

13 x

416 H ₂ O
130 GB
8 1 dNTP
3.25 FW
3.25 RV
13 poly
(1 template)

LI-YmlUG, SnRK2.8, AvrPto 2D → { SEVA T1 seqF
(Bp1 mut) O...R~~2. Sinf~~

33

23 x

736 H₂O
161 dNTP
230 GB
6.25 primers
23 poly

2. Yeast transformation to check localization (LEU)

pG CBL mcherry +
(HIS)

AvrPto	- mGFP
AvrPto(G2A)	
AvrPtoB	
SnRK2.8	
BIK1	
AHA1	
LUC	
EV	

3. make new plates, propagate some plasmids
spec, gent

40

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	15.6.2023		

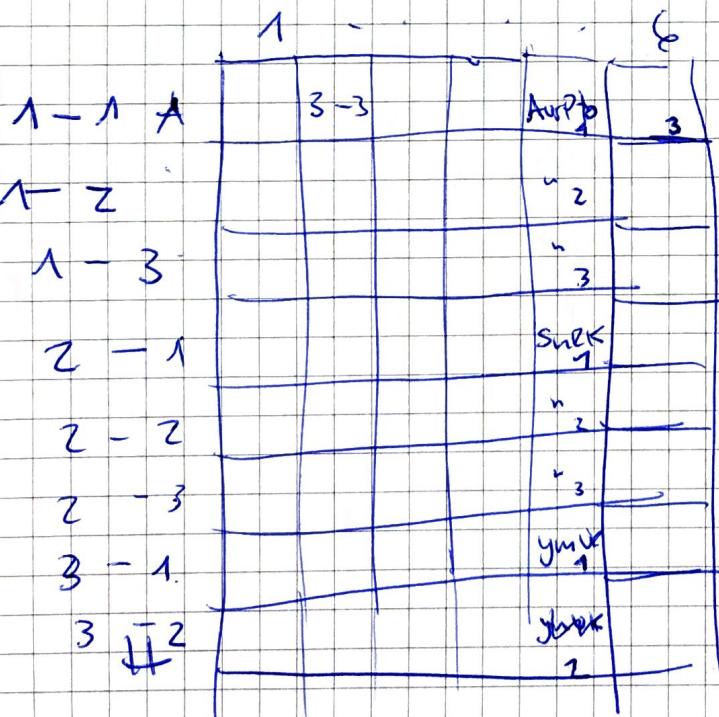
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4. To construct pGBT7-AvrPtoB (F749A) }
 ① pGBT7-AvrPtoB (F749A)
 ② pACT2-CERK1 cyto-PTAP } for interaction validation
 and roY2H
 ③ pACT2-SnRK2.8-PTAP
 ④ p641-HopA01
 ⑤ FRP795-ymLLKG1 (replace mCitrine by ymLLKG1)



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5 mini prep

PROJECT CERK1 FL

1-5
2-3
2-4

S8's

31 1-1
2-1p641 - AvrPto - 2, 4
2DGa 3-1
4-1p641 - SnRK2.8 - 2, 4
(BA mutant)Gb 5-1
6-1

p641 ymUKG1 - 2, 4

Vv 7-1
8-1AvrPto - Nub - 1
(WT)XL 9-1
10-1

G2A

:

S46P

:

Y89D

:

S46P/Y89D

PCR 25x 12x 32 = 384 H₂O

10	120	9B
7	84	dNTP
0.2	2.4	Taq
0.2	2.4	KV
1	12	Polymerase

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• prepare plates, etc.

5 4% Agar 50ml into 250ml bottle. x 6

10 liquid LB 500ml 200 ml in 250ml Bottle x 4

15 Solid LB 500 ml in 1L Bottle x 2

20 18.6.2023

~~Keep 2023 JJA~~

Re-miniprep AmrMo-Mnb ②,③
S46P/Y89D

25 digestion test. (15μl)

~~AmrMo~~
AmrMo

30 ~ 1 μg DNA (can be less, 500 ng should be also enough)

35 10X Cutsmart buffer → 500 μl / 15 μl

(EcoRI, NheI) enzymes(s) 0.75 μl each

40 to include:

- positive control !

- undigested group !

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	2	2NC	3)	3NC	PC	PCNC	
5	Buffer cutSMART /			1.5 μL *			
Eco RI		X		0.75 μL *			
Nhe I		X		0.75 μL *			
DNA	6 μL	6 μL	5	5	6	6	
water	6	7.5	7	8.5	6	10.7.5	

→ Reconstituted primers 21 - 32

→ ALL 3 look good

yeast transformation

BAK1-Cub + { AarPto-Nub } WT
 S46P
 Y89D
 S46P/Y89D
 G2A
 CBL-LUC-Nub
 n12
 LUC-Nub
 BIR2-Nub

AHA1-Cub + { AHA1-Nub
 BIK1-Nub
 BIK1KD-Nub
 LUC-Nub
 CBL-LUC-Nub }

MIR2-Cub + { PER1-Nub
 BIK1-Nub
 BIK1KD-Nub
 LUC-Nub
 CBL-LUC-Nub }

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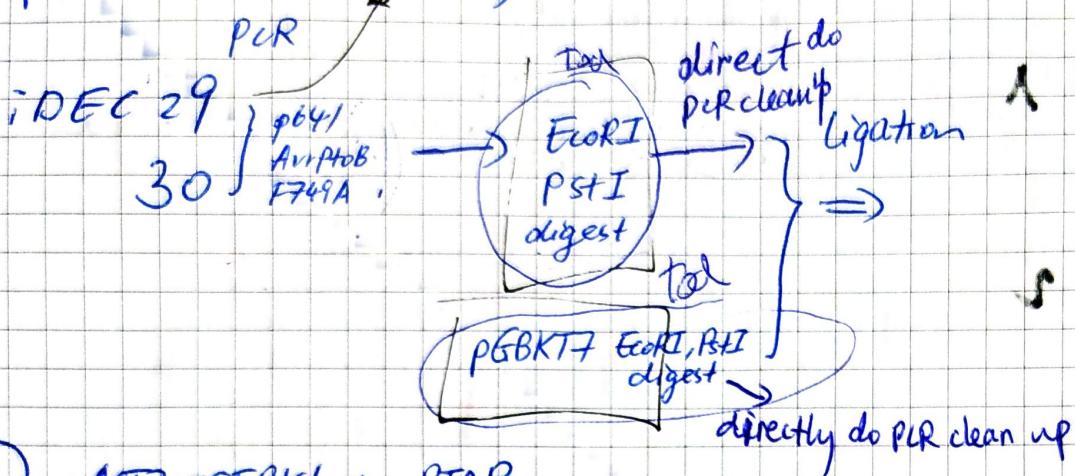
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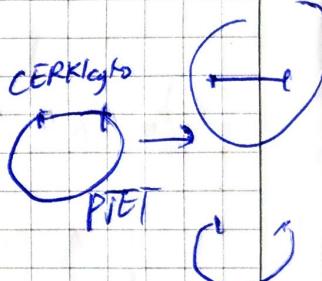
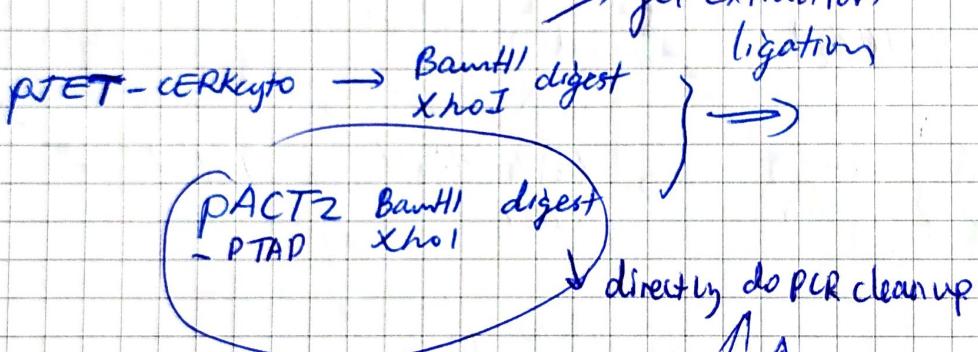
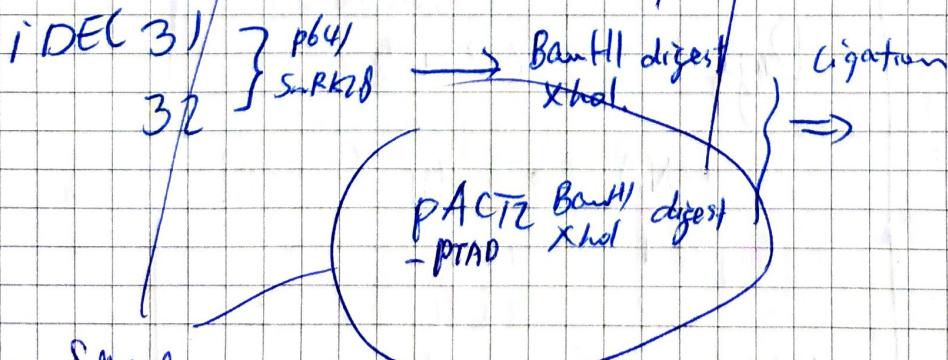
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p45 cloning

① pGBTKT7-AurPhoB(F749A)



② pACT2-CERKlayto-PTAP

③ pACT2-S_nRK2.8-PTAP

Same

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(4) p641-

HopAO1 \leftrightarrow (BpI mutated)iDEC 21 }
24 }

1

iDEC 23 }
22 }

2

BpI Golden gate cloning

Backbone p641

- calculate molar concentration
 $\sim 1:3$ ratio

matrine

(5) FRP795-ymuKG1

(integration plasmid
(lexop)-ymuKG1)

PCR clean up

iDEC 25 } p641
26 } ymuKG \rightarrow EcoRI digest
XbaI

ligation

FRP795 EcoRI
XbaI digest

gel extraction

LB plates

10x Cabs

10x Spec

10x Grea

10x 5x Kan.

Scale up

Digestion (20 μl)

DNA \geq 2 ng (2000 ng.)

Enzyme 1 1.5 μl

... 2 1.5 μl

10x Cutsmart buffer 2 μl

ddH₂O to 20 μl

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cab

kan

20.06.23

PCR clean-up a^t:

- Avr PtoB (idec 29 + 30)
- SbRlx2.8 (idec 31 + 32)
- GFP⁺gfp⁺ (idec 25 + 26)
- HopA01 (idec 21 + 24)
- HopA01 (idec 23 + 22)

- ①
- ③
- ④
- ⑤

Start pACT2-PTAP overnight culture. (3 tubes) (6ml)

Start FRY70 overnight culture (2 ml)

Digestions:

- | | | | |
|--------------------|---------|---|------|
| • Avr PtoB (F749A) | → EcoRI | + | PstI |
| • PGBKT7 | → EcoRI | + | PstI |
| • CERK1-cyto | → BamHI | + | XbaI |
| • SbRlx2.8 | → BamHI | + | XbaI |
| • pACT2 - PTAP | → BamHI | + | XbaI |
| • P041 ymUkgI | → EcoRI | + | XbaI |
| • FRP795 | → EcoRI | + | XbaI |

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21.6.23

→ Miniprep PACT2-PTAP plasmids - run out

→ PCR cleanup : AvrPTOB 1 50
 pGBKT7 2 50
 PACT2-PTAP 3 50 Digests
 YmVJK 4 50
 SmRN 2,8 5 50

run on a gel with pJET-CERK cyto digest
~~FOP795~~
 gel extract

→ Golden gate library can be transformed directly

make yeast plates ~ 50ml
 → Dropout - LWH

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- A Empty
 B control, A+A 1-4, A+Bik1
 C A+CBL-Luc 1-5, A+BKKD 1-9, A+LVC
 D M+cbl-cub 1-4, M+LVC 1-4, M+PEP1 1-4
 E M+Bik1 1-9, M+Bik1 KD 1-9, LucNub+Bik1cub 1-4
 F Bok1+Cub1 N12 1-4, Bok1+Bok, S46P1-4 } these are all
 G WT 1-4, S46P 1-4, WT 1-4 }
 H Y83D 1-4, G2A 1-4, S46P/Y83D 1-4 AvrPTO

FRP795 without methionine ~5500 bp 100 ng 35 ng/μl
 ~30 fmol

PGBKT7 EcoRI, PstI digest ~7300 bp 3 ml 27 ng/ml
 ~22 fmol

PACT2 XbaI BamHI digest ~8100 bp 4 ml 24 ng/ml
 DTAP ~20 fmol
 4.5 ml

AvrPTOβ 1667 bp ~15 fmol/ml 8 ml
 15 ng/ml

SmRK2.8 ~1000 bp ~40 fmol/ml 3 ml
 25 ng/ml

CERK1 cyto ~1000 bp ~50 fmol/ml 3 ml
 31 ng/ml

HopA01 ~750 bp ~40 fmol/ml 3 ml
 15 ng/ml

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$$\begin{array}{r}
 16 \\
 5 \\
 3.5 \\
 0.1 \\
 0.1 \\
 0.5 \\
 \hline
 8.00 \\
 2.50 \\
 1.75 \\
 0.5 \\
 0.25 \\
 \hline
 2.5
 \end{array}$$

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23.6.2023

→ Lianda PCR on tubes!

H ₂ O	32	3x
5×green	10	101.4 ✓
dNTP	5	30 ✓
RW	1	15 ✓
RV	1	0.3
x7poly	1	0.3
		3

→ Confirm the HopA01
plasmid before mix prep
(stroms formed on Wed)

p641

Goldengate

① AvrPTO2D-Nub → phosphomimic for AvrPTO → see fimbriotaxis more strongly

② FLS2-Cub better bio plasmid so you have low bg

dest	prom	N	Gem	C	Term	Aft
① Lev	PADH HD (middle)	dummy (BB00)	AvrPTO 2D	Nub	ADH1	dummy

② HIS	SH (short)	SP	FLS2	Cub	ADH1
	PADHI	HA	~sig		

also truncated ADH1 → weaker yeast signal → better secretion & localization to membrane
but stronger No plmt sig peptide

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MD - no conc \rightarrow 1 μ LRecepie \rightarrow from page 87

	Dest	prom	N	Gene	C	Term	Aft
Aur	0.5N	0.5 ✓	1. ✓	1.8 ✓	0.6 0.8 0.7 0.8 ✓	1.5 ✓	1.5 ✓
FLS	0.7 ✓	1.2 ✓	0.7 ✓	1 ✓	0.6 ✓	1.5 ✓	1.5 ✓

Aur FLS

7.156 ✓	7.2 ✓
1 ✓	1 ✓
0.5	0.5

BsdI 0.5 0.5

Bsr 9.2
9.0D_{H2O} 0.4 ✓ 0.8 ✓25 cycles standard
protocol

p

 \Rightarrow Transformation

DH10b ✓ important

GBKTF - Km

Act2 }
F2P } Comb \rightarrow transform some 10ng a bit of
m_g \rightarrow All gent \rightarrow 28 for 1 transform \rightarrow 1st
2nd

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EBK PTOB

A1 - B4

ACT
CE2K BS - D2ActSmRN D3 - E~~1~~ 6

AvrPbB-Nub F1 - S

→ Light ~ g 1 (2 = control (pos.))

Vector FCP 785 5500 bp 100 mg 4

6

Need 68.18 mg of insert for

S:1

(2.5) μL.

ligation

vector 3.5

insert 3.5

Lig buff 1

Lig 1

water 1

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2023.6.26

Sequencing alignment.

pGKKT7 AuroB F439A (3) (6)

pACT2 CERK1 PTAP (4) (8) ~~cyto~~pACT2 SnRK2.8 PTAP (2) (5) ~~cyto~~

{ collect } 3mL

AuroB Nub

(1) (2)

p641-SnRK2.8 (BpiI mut)

(2) (3) ? SIN?

2023.6.27

miniprep BB6b 1
BB09 2

pADHMD 3

CJ B 4

150 h

NUB 5

ADU Ter 6

FLS2x 7

SiFLS 8

FRP795 digest EcoRI xhol → PCR clean up

Bgl II
Mfe I

for ligation. add excess amount of yeast kG1

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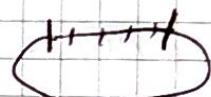
DATE

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26.6.

① FRP795 digest with EcoRI → XbaI → PCR clean up
add excess amount of gmluKG

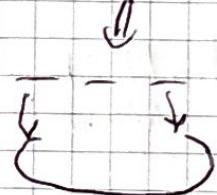
② digest with EcoRI, XbaI, MfeI ...



BstXI 3.1 100% Cutsmart 25% 0.5

BglII 3.1 100% Cutsmart <10% 0.5

MfeI / MfeI-HF 3.1 <10% Cutsmart 100% 1 μl



EcoRI HF 1 μl

XbaI 1 μl

10 μl

~~vector~~
~~vector~~

GD

5.2 vector

gme enzyme

4 CutSmart

18.2

26.8 water

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A AHA1-mGFP mew 1-3, AHA1-mGFP + CBL-mcherry 1-3

AurPTO-mGFP + CBL-mcherry 1-3, AurPTO-mew-mGFP 1-3

B EV 1-3, BIK-1 1-3, Luc 1-3, SMRK2.8 1-3

C AurPTO B 1-3, AurPTO G2A 1-3

26.6. summary

Miniprep Golden gate components

Yeast localization cultures

Short restriction for FRP795

Short FRP795 OVR216 liquid cultures

27.6

- Check localization by confocal microscopy
- yeast transformation

OVR216

pACT2-PTAP + pGBT7

pACT2-PTAP + pGBT7-AurPToB F479A

pACT2-SMRK2.8-PTAP + pGBT7

pGBT7-AurPToB F479A

pACT2-CERK1cyto-PTAP + { pGBT7

pGBT7-AurPToB F479A

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SIGNATURE

pGBT7-AgT + pGBT7-p53

DATE

(PC)

DATE

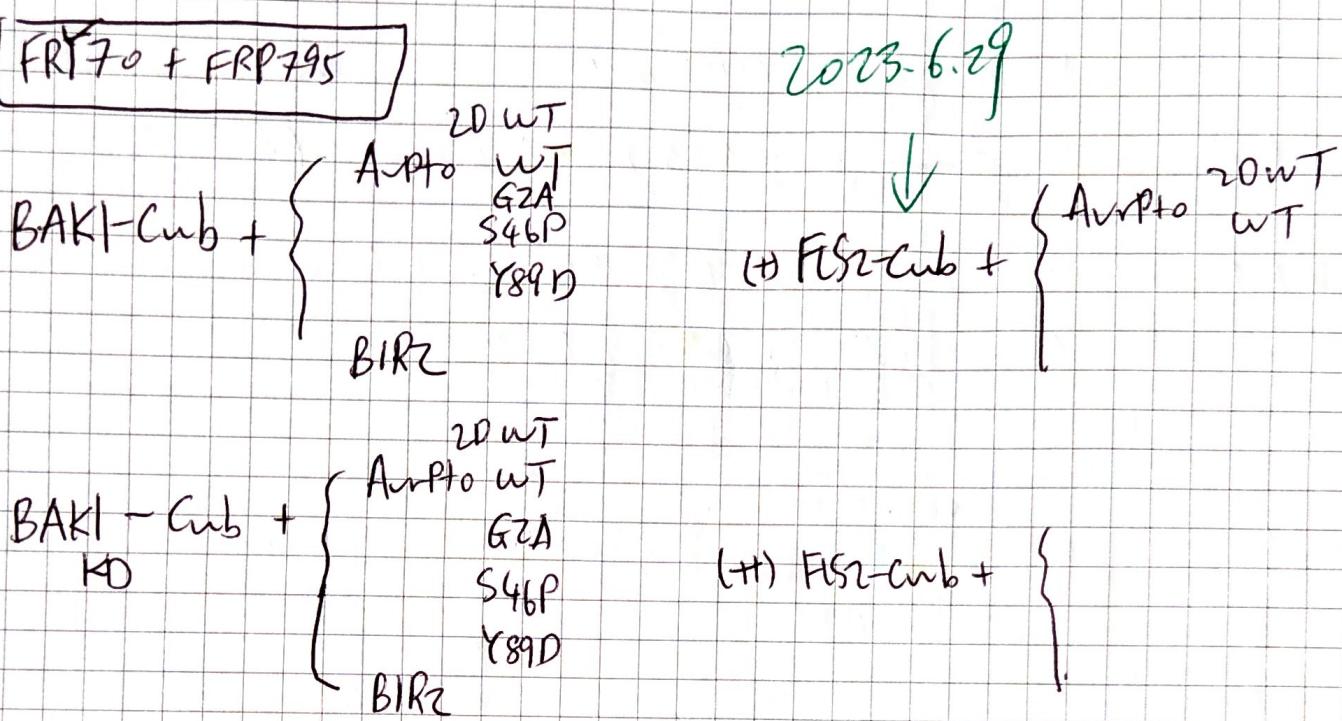
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2023-6-29



IDEC 37 592.4

38 551.5

39 577.4

40 559

Tomorrow

1. AvrPROB 3D Minprep + Sea

2. CE2N AvrPROB / SNR2-3 AvrPROB

Nr on cobases are on

→ Let grow & check

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Quicks Y2H

OD 0.2 → ~ 40/30_uL resuspend colonies
 in the water

↓
 you
 won't
 this

Multiple 3-5 colonies
 It's fine you
 will readjust
 Average size this later
 colonies

Started forward Y2H on 1.7.2023 rep 1

2.7.2023 rep 2

reverse . . . 3.7 . . .

PGBKT7

PACT2-PTAP

1. EV EV

2. AvrPhB F479A EV

3. EV CERK1 cyto

4. AvrPhB F479A CERK1 cyto

5. EV SmRK2.8

6. AvrPhB f479A SmRK2.8

7. Positive control AgT + p53

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Air - 22°C 2.5 A V 2 F 2.6
 0.9 SNR 28 0.6
~~7/2 2023~~

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13.7.23'

5 10 μl Green Buffer
 5 μl dNTP
 1 μl PW
 1 μl REV
 1 μl Poly
 ~10 ng Template
 32 μl H₂O

- 1 ✓ CE2C1
 2 SNR 2.8
 3 Air
 1 SNR 2.8
 2 CE2C1
 3 AmprOR-F
 4 AmprOR-D

20 13.3.3 F2P7GS + μM UK GB

25 5.102 F2P7GS

PciI restriction

Template
 - rcat SMRT
 Enzyme
 Water

F2P7GS
5.1 ✓μM UK GBS
13.3 ✓

Digest

H 4

H 4

7 GBS 23

H 4

H 4

2 Air - 5 38
3 Air - 0 22

3 H 4

3 H 4

4 ACT2 - 32
5 9357 > 10 1 (SNR)
6 91347 > 10 2 (CEN)

26.9 ✓

18.7 ✓

BamHI rcut smart 50%
 12.1 100%

HF is in
 rcat smart

EcoRI BamHI NcoI SAI I
 NciI XbaI

EcoRI + NciI

~~EcoRI~~
 SAI 3.1
 NciI 0.11 (0.0)%

XbaI treatment 3.1, 2.1

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~~500 μL 1% Biotin → 10 μL same dilution +~~

~~0.5 × 20 μL~~

~~1 μL same dilution 200 ·~~

~~10 μL 100% EtOH + 10 μL H₂O + 10 μL 3M NaAc~~

~~10 μL H₂O~~

~~10 μL 3M NaAc~~

16.07.2023

COLONY PCR

A1-H1 → AVRPTOB F473A3D (1-6) + AVRPTOB F473A (1-2)

A2-F2 → P38K-CERK1 (1-6)

H2 → PC - ~~pACT2~~ pACT2-CERK1-PTAP

G2 → PC - pGBKTF7-AVRPTOB F473A

PRIMERS USED F R

FOR AVRPTOB → iDEC10 S2216

FOR CERK1 → iDEC41 S2216

GEL LOADING

LADDER	EMPTY	A1	B1	C1	D1	E1	F1	G1	H1	EMPTY	G2
--------	-------	----	----	----	----	----	----	----	----	-------	----

LADDER	LADDER	A2	B2	C2	D2	E2	F2	EMPTY	H2
--------	--------	----	----	----	----	----	----	-------	----

↑ ↑
THEY FAILS

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~~1) Turbos until 3D~~

- PCR Turbos until
- Trimming down smaller to 3D
- PCR or cDNA Vigs

A 20.7

PCR of cDNA for Vigs

0.5 μl goTaq polymerase ✓

0.25 μl F primers

0.25 μl R primers

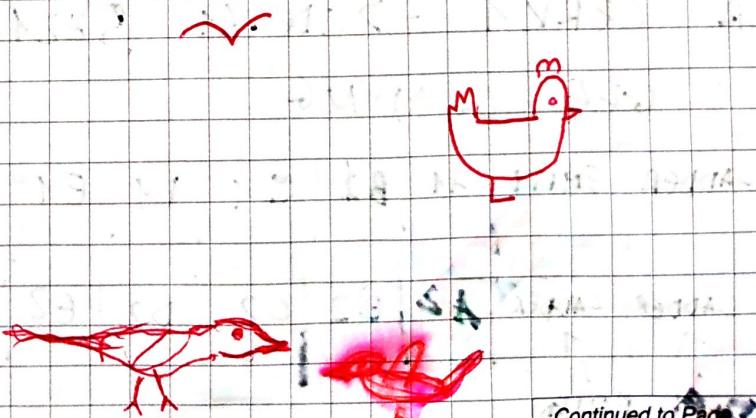
10 μl 5x goTaq buffer ✓

10 μl dNTPs ✓

1 μl cDNA ✘

27 μl ddH₂O ✓

1 μl ✘



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PCR for Gibson Ass.

1+3, 2+4

1. p53 - GSG - E2A template: pJBKT7 - p53

i) DEC51 MJ 1030 bp → 6
ii) DEC52

2. p53(65)3 template: pJBKT7 - p53

i) DEC51 1002 bp
ii) DEC53

3. E2A Bleo-2 template: pPIC2A

i) DEC54 445 bp
ii) DEC56

4. p53(65)3 template: pPIC2A

i) DEC55 424 bp
ii) DEC56

24. 2. 23

Colony PCR screen 2-3

PCR Aw PtoB

1: AupbtoB F479A

1: 63.6 ng

2: AvrPtoB F479A 3D

2: 61.92 ng

3: 47 ng

4: 26.70 ng

(Gibson Assembly)

Optimal

Inserts

0.1 μ L - 7019

Vector

0.1 μ L - 70979

MM

10 μ L

-

H₂O10 μ L231137
0320

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276 μ l H ₂ O	300
138 μ l gB	150
80 μ l dNTP	87
345 FW/RV	3375
11.5 μ l PstI	12.5
13 μ l colony)

5

10

28/7/23

Yeast transformation

15 1. pGBTKT EV + pACT2 EV ✓

2. pGBTKT EV + pACT2-AvrPtoB F479A (= mut) ✓

20 3. pGBTKT EV + pACT2-AvrPtoB F479A 3D (-3D)

25 4. pGBTKT - SmRK2.8 + pACT2 - pACT EV (mut) NO

5. pGBTKT - SmRK2.8 + pACT2 - AvrPtoB F479A (= mut)

6. pGBTKT - SmRK2.8 + pACT2 - AvrPtoB F479A 3D (-3D)

7. pGBTKT - LERK1 + pACT2-EV

8. pGBTKT - LERK1 + pACT2 - AvrPtoB F479A (= mut)

9. pGBTKT - LERK1 + pACT2 - AvrPtoB F479A 3D (-3D)

10. AvrPtoB mGFP 2160 PEG

3 mL PEG

11. SmRK2.8 mGFP 360 LAC

500 mL LAC

450 ssDNA

630 mL ssDNA

40

pACT - LEU (L)

pGBTKT - TRP (W)

45

PLATES USED - LW

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30/07/23

MINIPREP

PACT2

PACT2-AVRPTOB FG73D (4)

PACT2-AVRPTOB FG73D (3)

PACT2-AVRPTOB (4)

PACT2-AVRPTOB (6)

mg/ml

38.3

145.2

46.0

43.4

50.3

1 pgBKT + EV + pgADT-T /

(2) pgBKT-p53 + pgADT-T /

(3) pgBKT - p53 - E2A - B10R + pgADT-T

~~(4) pgBKT - p53 - unk - B10R + pgADT-T~~

pgBKT EV + pgADT EV

pgBKT-p53+ pgADT

pgBKT - p53 - E2A - B10R + pgADT EV

~~(6) pgBKT - p53 - unk - B10R - B10R + pgADT EV~~

4x

$$\$ \times 240 = 1200 \text{ PEG} = 750 \text{ 80% PEG} + 450 \text{ water}$$

$$\frac{200}{250} \text{ UAC}$$

$$\frac{200}{250} \text{ ss}$$

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1) CERK +

2) CERK NEW

3) EZA

4) p 441

£.88

£.211

0.11

£.44

£.02

FEB 1985

SUNDAY

STDA

(1) 10519 80199VA - STDA

(2) 10519 80199VA - STDA

80199VA - STDA

80199VA - STDA

5

10

15

20

30

35

40

45

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4. 8

① Transformations

- | | | |
|----|-------------|-----------------------|
| 1) | pGADT7 | + pAct2 |
| 2) | " | + pAct2 - AcrPtoB mut |
| 3) | " | + pAct2 - AcrPtoB 3D |
| 4) | " - SfRK2.8 | + pAct2 |
| 5) | " - SfRK2.8 | + pAct2 - AcrPtoB mut |
| 6) | " - SfRK2.8 | + pAct2 - AcrPtoB 3D |
| 7) | " - CERK1 | + pAct2 |
| 8) | " - CERK1 | + pAct2 - AcrPtoB mut |
| 9) | " - CERK1 | + pAct2 - AcrPtoB 3D |

② mini prep of ~~transfected~~ pGADT7

Plan 5.8: (or later)

- Transformations w/ pGADT7
- insert CERK1 into backbone
- amplify pGBKT7-E2A-BleoR & pAct2-E2A-BleoR

5/8

Tzafos

n

pGBKT7

- 1 EV /
- 2 p53 /
- 3 p53-E2A-BleoR /
- 4 p53-GS-BleoR /
- 5 EV /
- 6 p53 /
- 7 p53-E2A-BleoR /
- 8 p53-GS-BleoR /

pGADT7

- | | |
|----|---|
| T | / |
| T | / |
| T | / |
| T | / |
| EV | / |
| EV | / |
| EV | - |
| EV | - |

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6/8

5 PCR ~~overnight~~ E2A-BLeoR (primers 57-58)

Oligo dT VIGS LAMP

10 PCR → 1: CEFK12 (ca. 1.3 µg) LAMP → CEFK12 Express 2 µL
2: TKV (ca. 2 µg) TKV: 1 µL

15 9/8 colony PCR

positive: 1.7 kb.

negative: 1.2 kb.

8.2

11/9

BLeoR E2A-BLeoR (PCR)

RT for pGBKT7 and pACT2 and pADT7
via primers 57-58

1 → for split ubiquitin

1 → E2A-BLeoR (pGBKT7, pACT, pADT7)

2 → E2A-BLeoR (Lub/Nub)

3 → (IS)-BLeoR (Lub/Nub)

4 → Lub

5 → Nub

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Miki paper

- 1
2 Extraction 1,2
3
4 Gus E2A BlaR 1,2
5
6 Gus GUS BlaR 1,2
7 Nob E2A BlaR 1,2
8 NUS GUS
9 Nob E2A BlaR 1,2
10 Nob E2A BlaR 1,2
11
12 Gus UXL 1,2

Colony PCR

- A: pTRV2 - NUCOMK1 B: pGIBRT7 - GUS - E2A - BlaR
 C: pGADT7 - GUS - DTA - BlaR D: pART2 - GUS - E2A - BlaR
 E1: pTRV2 E2: pGBKT7 E3: pGADT7 E4: pART

Agrobacterium GV3101 - Rif/Cen resistant
 pTRV2 - Kanamycin resistant]
 ↳ LB-Rif/Cen/Kan.

vector construction

- 1 pGAL1-gusmk1 - NotI
 2 FRP795 - PacI
 3 FRP795-gusmk1 } }

ADT2 PCR

- 1 - pGADT
 2 - pGADT AT
 3 - pGADT AT

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5 - LWW
drop out by filter

10 Rif - next - Van plates for Agar.

125 mL medium

125 μ L Van
125 μ L next
25 μ L Rif.

minimum viable

100% viability

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21.8.2023

yeast transformation

pGBKT7 pACT2

① EV + EV NC

② p53 + AgT PC

③ ArrPtoB + EV
F429A

④ EV + CERK1

⑤ ArrPtoB + CERK1
F429A

Direction 1

⑥ CERK1 + EV

⑦ EV + ArrPtoB
F429A

Direction 2

⑧ CERK1 + ArrPtoB
F429A

3D's effect.

⑨ CERK1 + ArrPtoB F429A
3D

ArrPtoB F429A

ArrPtoB F429A 3D

} for directed evolution

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⑫ pGALL-ymuket1 (Not I. digested)

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empty vector

①

pGBTKT7-G5G-E2A-BleoR
 pGADT7-G5f-E2A-BleoR

BamHI

SalI

[GS linker ...]
 GS-E2A-BleoR

pGBTKT7-p53-

pGBTKT7-BamHI-SalI

pGADT7-BamHI-XbaI

②

P641-(lexAop)₈-Percimin

FRPZ95

FRPZ95-gmllKG

oligo annealing

left adaptor
right

EcoRI, NotI, FRPZ95

→ 2 h digest

→ 3 µg of DNA

→ 1 µL of each enzyme

Buffer

GSLink
BHKE2A
AMM

Bath

+ Cut Smart

BamHI

BHK

3

F2P

3

SalI

BHK

1.5

EcoRI

water

BHK

8

1.5

NotI

DNA

BHK

16

14

10

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→ Dissolve & anneal the oligos

64 left F 1194.6

63 left R 1033.9

66 right F 589.5

07 right R 600.5

10mM TEGS pH 7.5-8 (50mM MgCl₂) / nTEGTA

(10mL)

100 μL 1 M TEGS

100 μL 3 M NaCl

20 μL 0.5M EDTA

g.: 780 μL H₂O

→ 10 μL each + 80 μL buffer

→ 95°C, 3-5 min, let it cool

H ₂ O	32 μL	✓
S.BUFF/TEG	10 μL	✓
NTP	5 μL	✓
FW	1 μL	✓
EV	1 μL	✓
Poly	1 μL	✓
~10mg temp		✓

③ p64I - ura3-linker-ymuKGI

iDEC 69 } T_a = 56°C
70 } ura3

68 } T_a = 56°C
71 } ymuKGI

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1st Dec 2018

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① ~~①~~ Est: AvrPtoB

- Run digestion test
- Agro transformation
x 4

Rf+gent+spec

② Miniprep pGBKT7-E2A-BleoR x 2
(GS)
(out of frame)

Send
sequencing

③ p641-CERK1. WT (Opiz-mut)

2 frags 7/2
3 7/2
3 7/2
4 7/2

CERK1 K350N

CERK1 D441V

CERK1 K350N D441V

④ LII

Split ubiquitin Y2H E2A-BleoR
(GS)-BleoR.

BAK1-Cub

- Cub-E2A-BleoR

... - GS - ...

BIR2-Nub

Nub-E2A-BleoR

.. (GS) ..

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- 5 CERK1 WT → (72, 75), (74, 73)
 10 CERK1 K350N → (76, 77) (72, 75) (77, 74) (76, 73)
 15 CERK1 D441V → (72, 75) (74, 79) (78, 73)
 20 CERK1 K50N, D441V → (72, 75) (76, 79) (74, 77) (78, 73)

Reaction

			100 fmol
1	72, 75	561 bp	0.5
4	74, 73	1342	2 μl
9	77, 74	544	0.5
4	76, 73	838	0.5
5	74, 79	817	4 μl
20	78, 73	565	0.5
7	76, 79	278	1
8	78, 73	565	1

100 fmol
 1 μl
 2 μl
 0.5 μl
 0.5 μl
 4 μl
 0.5 μl
 1 μl
 1 μl

BB: 173 ng/μl ~50 fmol/μl

100 fmol
 1 μl
 2 μl
 0.5 μl
 0.5 μl

50 fmol

CERK1 WT: 0.5 μl W + 2 μl (2) + 0.6 μl BB
 CERK1 K350N: 0.5 μl (1) + 0.5 μl (3) + 0.5 μl (4) + 0.6 μl BB
 CERK1 D441V: 0.5 μl (1) + 4 μl (5) + 1 μl (8) + 0.6 μl BB
 CERK1 K50N, D441V: 0.5 μl (1) + 0.5 μl (7) + 0.5 μl (3) + 1 μl (8) + 0.6 μl BB

(+) always

0.5 μl Esp3I
 0.5 μl T4-ligase
 1 μl 10xT4 ligase buffer

ddH₂O to 10 μl.

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Infiltration
ago bacteria

- 5 1) spin @ 3000g, RT 10 min
- 10 2) prepare infiltration buffer, 10 mM of both compounds
 - $MgCl_2$ 1M \rightarrow 1:100
 - MES 0.5M \rightarrow 1:50
- 15 3) add 150 µL AS to 100 mL infiltration buffer

$$10 \text{ mL OD} = 0.5 \text{ samples}$$

$$\rightarrow 1:10 \text{ dilution}$$

~~0.125A9~~

$$F1: 2.2 \text{ mL}$$

$$F2: 2.54$$

$$1: 2.4$$

$$2: 2.35$$

$$P1S1: 0.43$$

+ Agarose • May 12, 1993

$$P1S7: 0.13$$

↓
35

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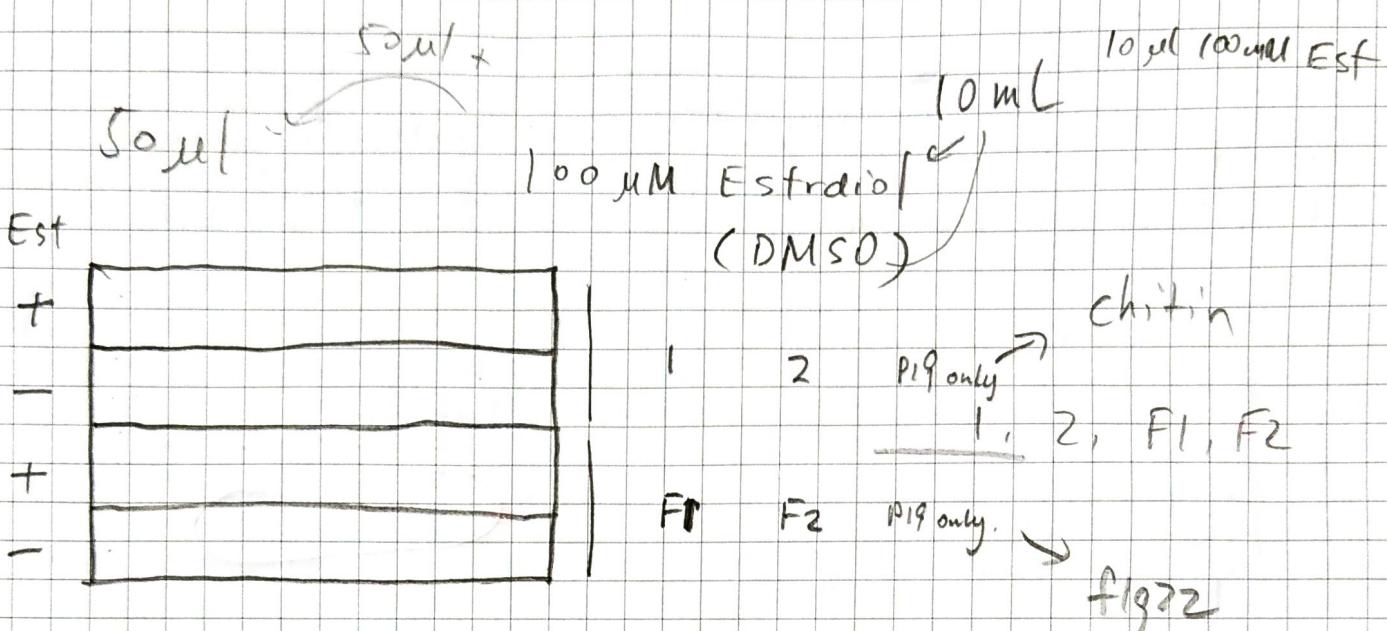
Continued from Page

dropout.

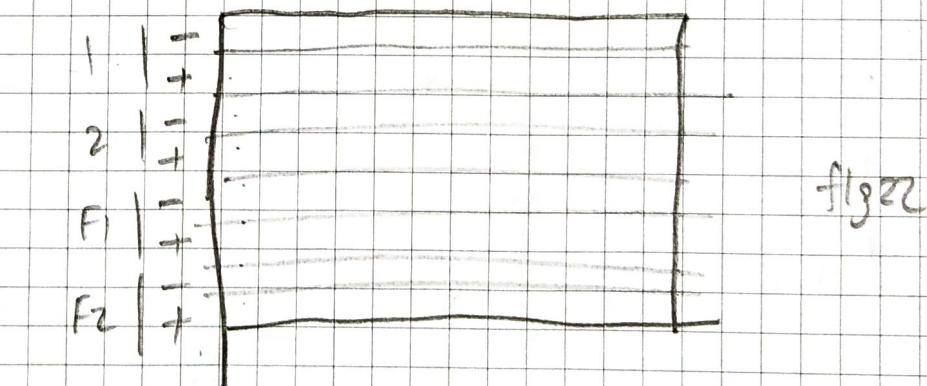
- leu liquid medium

→ for our Z16 transformed
with pACT2 AmP^RB

- LW plates → to repeat the SFA assay



100
Chitin
f1g22



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1) ROS assay

2) Cloning of CERK1 mutants

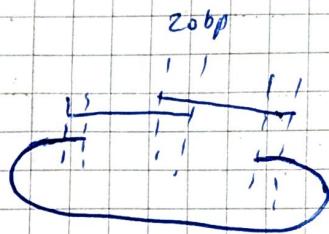
infusion

pJET CERKFL as template

V336M as an example:

digest pGFKT7

with NcoI, SalI

{ 87 e
 90{ 89
 88

7 mutations

K350N

D441V

K350N, D441V

87

88

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PEWIS

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30/08/23

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	A	1 248	2876
	V336 M:	87, 90	88, 89
5	L462S		
	3 b26	4498	
	87, 92	88, 91	
10	R474E:	87, 94	88, 93
	5 661	6 463	
	7 681	8 435	
	F480 K:	87, 96	88, 95
15	M483R:	87, 98	88, 97
	8 689	10 435	
	V488S:	87, 100	88, 99
	11 702	12 422	
20	F525T:	87, 102	88, 101
	13 814	14 310	
	K350N:	87, 88	1064
25	D441V:	87, 88	1064
	15 16		P641
	K350N, D441V:	82, 88	1064
30	(1)	67	
	R474E → A1-A7		32 dH ₂ O
	F480K → A8-A9		8176 256
35	(1) L462S → B1-B7		10 buff 720 80
	(1) V336M → B8-B9 C1-C6		5 NTP 360 90
	(1, 2, 3) 472, 350N → C7-C9 D1-D3		1 Fw 72 ✓ 8
40	(2, 3, 4, 5) 14 D591V D9-D9 E1-E4		1 Rv 72 ✓ 8
	(2, 3, 4, 5) 14 D591V K350N B5-B9 F1-F6		1 Poly 72 ✓ 8
45	(5, 6, 7) 1 V999K F7-F9 G1-G4		2304 ✓
	(2, 3, 4, 5) 1 M993R G6-G9 H1-H3		
	(1, 4, 5) 1 F525T H4-H8		
		PC HG	
			Continued to Page
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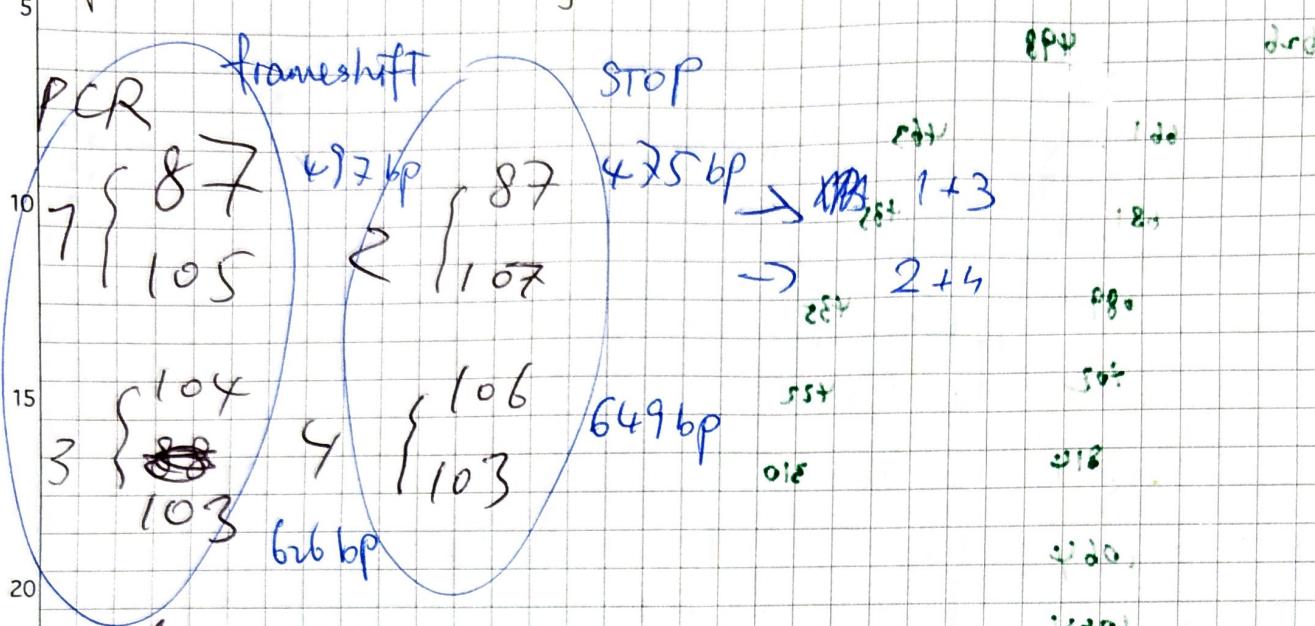
DATE

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358

835

primus 108 + 109 → colony PCR



digest pGKTK7-GSG-E2A-BleoR with NcoI, BamHI
 ~1500ng 20 μl 1 μl enzyme each

make a gel

Gelby PCR

suspensions: 1-16: 480 neat 1-16
 17-21: 336 neat 1-5
 22-24: 336 1-3

Column 1: 1-8
 2: 9-16
 3: 17-24

1-16 835 480

2H-1H 83 83 83

1-8H-2H 83 83

colony PCR in Blumro cycle 5, other in 2.

W2 GB 51.5 dNTP 25.5 phur.

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plates

- LW x 200ml

- LW

- LW 100ml

- LW

- LW + 2 * FoA

480

- LW + Zeocin

200 μl Green

✓ 210 μl Green

100 μl DUTP

✓ 110 μl DMTP

20 F

✓ 21 μl F 10g

20 R

✓ 21 μl R 10g

20 Poly

21 μl Poly

600 μl

672 μl ✓

A3

1000

A1 - C4 Positive: 9, 12, 13, 15

✓ 290 Green

190 DMT

2, 4

28 R

C5 - E4

✓ 28 F 10g

(6) X

✓ 28 P 110

480 nest

✓ 886 H2O

✓ ✓ ✓

E5 - H4

⑩, 11, 12, 13

12, No 3 K, S -

HS - PC HG - PC

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orange

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5

A B

84

BOOK

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8/1

PROJECT

3 5

4 9 9

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OD

 H_2O

5 Agro: 0.370 → 0.370

1.35 mL

P19: 0.391

1.96 mL

to 10 mL

FLAG: 0.431

4.31

4.06 mL

Na FLAG

4.33

1.15 mL

15 Vigs 3-14 4.25

~~2.737 mL~~

4.70 mL

to 10mL

PTCVI 0 4.47

~~2.0835 mL~~

4.47 mL

MQH₂O

Vigs 3-13 3.35

1.28 mL 5.97 mL

PTCV2-GFP 3.16

1.1 mL 6.33 mL

PTCV1 + 3.38

1.31 mL 5.91 mL

PTCV1 - 3.54

1.46 mL

5.65 mL

$$1.9 \cdot 4.25 = x \cdot 2$$

$$x = \frac{1.9 \cdot 0D}{2}$$

1trunc

1, 2, 11,

All

2mut

5, 6, 7, 9, 10, 12, 19, 15

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56 °C_{air}
50 μl yeast{ IDEC83
84 }Esp3^I-lacZ-Esp3^I → p_{B41}-Bpi^I 45 sec elong 750
X 18 34{ 72 6
75 2
74 3
85 3 }500
250p_{B41}Esp3^I

CERK1

c1

PJET CERK1 FL.

0.6 μl temp 4 μl ins

1 μl lig buff

1 μl Bpi^I EC⁴³

to 10 μl 52°C

Inserts: 1 μl
 X 38: 1 μl
 T4: 1 μl
 T4 ligase: 0.5 μl
 Enzyme: 0.5 μl 3pi-1
 H₂O: 0 μl

83/84	74/83	72/75
p01	p02	p02

 ESP3^I

yeast transformation

X 1.1	F88ST	①
1	L962S	②
1	M983R	③
1	R479E	④
2	F980K	⑤
1	V983K	⑥
1.7	V336M	⑦
1.2	D99Y K350N K350N	⑧
1.2	K350N	⑨
1	D99Y	⑩
2.5	WT	⑪
1.2	ZmUT	⑫

square plate

- LW

- LWL

- LW + FOA

Single mut x 7 ✓

350 ✓

441 ✓

350 441 ✓

Family

WT ✓

1.1	STOP	⑬
1.25	f-wif	⑭
2	EV	⑮

round

- LW

- LWL

- LW + Focin

STOP n p
frameshift ✓
EV ✓

ADHα1

- E2A BleoR

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15

WITNESS CERK

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TITLE Start directed evolution PROJECT

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primers

template

to which backbone.

5 iDEC (08, 109)

① pGK7-CERK1

pGK7

NcoI SalI
digested

10 (08, 110)

② pGK7-E2A-BleR
E2A-BleRpGK7-E2A-BleR
out of frameNcoI, BamH I.
digested

15 PCR using GoTaq

20 50 μL GoTaq system

Template	pGK7-CERK1	2.5 μL
5x GoTaq Buffer	10 μL	2 μL
Nucleotides	5 μL	2 μL
Fw primer	1 μL 2.5 μL (diluted)	1 μL
Rv primer	1 μL 2.5 μL (diluted)	1 μL
GoTaq Poly	0.25 μL	0.25 μL
	up to 500 mg	up to 500 mg
	to 50	to 50
	→ 32.75 μL	→ 32.75 μL

30 Backbone Digestion:

* 40 μL system: each

- 2 μL enzymes
- 4 μL template
- 1 μL H₂O
- 1 μL Buffer

→ put at 37°C

pGK7-CERK1

NcoI/SalI
12.9 μL
19.0 μL

pGK7-E2A-BleR

NcoI / ~~BamH I~~ BamH I
15.3 μL
10.7 μL

① digested 20 μL + 20 μL

② digested pGK7 only

③ digested pGK7 only E2A-BleR

10 μL

20 μL

④ digested pGK7 + CERK1

digested pGK7 + CERK1

10 μL

20 μL insert

①

digested pGK7 + CERK1 insert

②

30 μL

~ 1 μL backbone

~ 4 μL insert

vs

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734 bp p641 → 742

~~24~~ Remove BpiI sites from estradiol inducible promoter
template
for Cytobath

iDEC146 1 452 bp
iDEC112 1 711-355-LacZ-Noymer

27.13 ng

iDEC111 2 1381 bp
SC490 2 Est-Arrtob

58 °C

85-34 ng

SC491 2 1220 bp
iDEC 47

25.30 ng

to cons
pGBTKT7-
Cherry 2A/02A-BleOR

G1 87 } 56 pJET-CERK1. FP 56 °C 20y 100 2 8,1
02A 114 }
56 p641-Erb-E2A-BleOR 28y 1 61 °C
pGBTKT7-E2A-BleOr
+ template

G2 87 } 116 1125 bp 70y 0.5

P2A 115 } 56 442 bp 28y 7

5x0 ←
6 5x0 ←
N 5x0 ←
3

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round plates

- LW → efficiency $\times 6$

- LW ~~WT~~
+ FoA $\times 3 \rightarrow 30\text{ mL}$

- LW
+ FoA + ~~BlacR~~
~~zeocin~~ $\times 3 \rightarrow 30\text{ mL}$

- LW
+ 2x Zeocin $\times 2 \rightarrow 25\text{ mL}$
~~LW + Zeocin~~

(D25A)

12.9.2023

Test STOP, Trunc, EV's resistance
to zeocin
by directly do plate assay
after transformation

Est Pro Bpil mut
get extr
PCR clean up.

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DM fusion 24 peptides

1: P2A1 → 1 μL pGBKT7, 0.5 μL 4, 0.5 μL 5

2: P2A1 → 1 μL pGBKT7, 0.25 μL 6, 0.3 μL 7, 0.6 μL MQ

3: P2A2 → 1 μL pGBKT7, 0.5 μL 4, 0.3 μL 8, 0.2 μL MQ

4: P2A2 → 1 μL pGBKT7, 0.1 μL 6, 0.3 μL 9, 0.6 μL MQ

Yeast transformation

→ STOP →

→ truncated (deletion) 1.5 OD

→ EV (E2A Rho R) spin down

→ intact CERK in E2A Rho R

resuspend
in 0.05 ml

Yeast transformations:

DNA (1 μg vector + 4-5 μg insert)

* CERK1 (1.5 μl)

* NC (14.3 μl) } total cells

* CERK1-E2A-Bleo R (15 μl)

* NC (11.8 μl) } each

* STOP

* Truncation (2.3 μl)

5 μl each + H₂O
cells ↑
to 34 μl

* EV (3.8 μl)

~~Wash cells~~

* Intact (3.5 μl)

Colony PCR:

1) CERK1 T+E: 5, 8, 12, 13

2) lacZ: 1, 7, 8, 9, 10, 11, 4, 10, 11, 12, 13, 14, 15

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5. 1. Keep an eye on the yeast plates. (-LW)
10. 2. P2A ORA (mini-prep)
15. 3. On Thursday, brush estradiol (estradiol + silent) 50 μ M 0.04% (1/2000)
middle vein as border
or indicate border
- 20.
25. 4. Redo lacZ construction
on page 85 template LJ 35S lacZ mGFP Noster
30. BpiI goldengate to 4174 pb41 BpiI (pol)
ApL 3B
35. 5. p87 estradiol (BpiI removal).
to pb41-ESP^{3I}_(pol) Esp^{3I} goldengate. ApL BB 372ng insert
40. 6. VIGS inflorescence GFP first (NC)



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Galdengaste

(4.9 kb)

* ① + ② + ③ → p641 - ESp
 (452 bp) | 1318 bp | (1220 bp)

Esp 3I

54.35 ng	158.5 ng	146.7 ng	→ 1 μl	total: 4.75
0.65 μl	1.2 μl	1.3 μl		H ₂ O: 3.85

* LII35S LacZ-mGFP → p641 - BpT 3p

~ 500 bp

(4.9 kb)

~ 60.12 ng

1 μl

3.9 μl

total 4.9

H₂O: 3.1

1: Extractor 1: clean up LacZ

2: Extractor 1: clean up

3: Extractor 2: gel-extraction

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FRY70

5 - FRP795 x 3

FRY70 - FRP795-gmukG x 3

10

OUY216-pGAL1-gmukG x 3

15

OUY216-AvrPtoB x 1

20

2000 PCR

→ For 50 μL

↳ 12 μL Buffer

↳ 7.5 μL dNTP

↳ 0.25 μL undiluted primar

30

35

40

45

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Western blot!

1.5x dNTPs

0.25 μl each undiluted primer

PCR colony

→ 10 μl Buffer 100 ~~160~~ 21 12 each side

~~10~~ 7.5 μl dNTPs 75

0.25 F prim 2.5

0.25 R prim 2.5

1 poly 10

~~3.5~~ yeast 0

28 H₂O 280

40 °C

✓ +

30 °C

108/110

~~88~~ 25 μl

10 130

5 ~~65~~ 65

1 13

1 13

1 13

32 ~~16~~ 351

12.1

11.0

10.5

3

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5 1. prepare for the WB tomorrow

— inoculation

— WB gel

10 2. yeast miniprep. — inoculation — compare
BLEO vs nothor

15 3. miniprep

20 4. check VIGS plant

25 construct

30 5. plant CERK vector

p641-CERK (ECO+TM)-cc1

φ 1 ~ ~ [lacZ] - c10

35 MM1: SEVA

40 MM2: Lab stock

25

1.5h

Cold
room

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WB ODs

neg. → 5.4

OD 2.5

[2 → 4.685]	C-321	→ 1.077 ml
[3 → 2.155]		→ 1.16 ml
[5 → 3.5]		→ 0.714 ml
[6 → 2.9]		→ 0.862 ml
[7 → 4.65]		→ 0.537 ml
[8 → 3.31]		→ 0.746 ml
[10 → 4.04]		→ 0.619 ml
[12 → 3.66]		→ 0.683 ml
WT → 2.28		→ 1.096

BB06 BB08 Noster BB09

- 35 S promoter CERK1 [lacZ]
S. 15235 WT

• Est. promoter CERK1 [lacZ]
[lacZ]

CERK1 WT
CERK1 KD

Native CERK1 pro

→ 150 ng of each component +

For **1** reactions:

MM * 8

1.5 μl	T4 ligase Buffer
1.5 μl	T4 ligase
0.75 μl	SSAK
0.5 μl	ddH ₂ O
1.5 μl	LII
2 μl	BB06
2 μl	BB08
2 μl	Nos

1.5 μl 35S + 0.5 μl Est promoter

1.5 μl CERK1 ECM + TM + 0.4 μl CERK1 WT + 0.5 μl KD

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21/09:

- 5 1) Inoculate new cultures for WB (2, 3, 5 ...)
- 10 2) Night-seq for Goldie's gate from yesterday
- 15 3) Mini-prep inoculation - DE + plated
~~PGK72 CERK1~~ own + Vector
 - LW + SfOA + Zeocin
- * Only the E2T was transformed into
 E. coli → unsuccessful yeast mini-prep
 → 3 plates, 20 m total

20 Check the identity of these plasmids

25 pgk72 (6S_r)-BleOR

30 with CERK1?

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1) 117 + 119

μM 12

100 μM

Phusion PCR
muc.

- 1) 117 + 119
- 2) 117 + 119
- 3) 87 + 119
- 4) 119 + 56

| no CERK1 } T4 PNK
 | CERK1 } infusion

26/9 PCR → 1 - EV
 2 - un intact
 3 - stop
 4 - trunc KIAN

5) 117 + 119
→ annealing1) CERK1 trunc
temp 63°C, elongation 2.5 minutes

Reaction

1) no CERK1

2) (117) CERK1
3) (117+119) CERK1 trunc.
4) (117+119) CERK1 stop.

- 1) no CERK (117+119) (1)
- 2) CERK1 (117+119) (10)
- 3) CERK1 trunc. (117+119) (11) stop
- 4) CERK1 stop (117+119) (12) trunc
- 5) CERK1 trunc (87+119) stop
- 6) CERK1 trunc (118+56) stop
- 7) CERK1 stop (87+119) trunc.
- 8) CERK1 stop (118+56) trunc.

μMA → 117 + 119 μMB → no primers

EF-PCR mutation results

- * S510P
- * L398P
- * G502E
- * S444F
- * G481R
- * T367T + (silent)

- (17)
- (18)
- (19)
- (20)
- (21)
- (22)

- W412*
- (stop)

- F 267 L + L363Q (23)
- G569R (25)
- Myc-tag + L388* (stop) (26)
- C381W (26.1)

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5 Amplify native CERK1 promoter from AT gDNA

10 ① 120, 121 569 bp — gDNA

(~~goTaq~~ + x7)

~~goTaq~~

x7

15 ② 124, 125 1829 bp — 3'SS-CERK1-[x7]
(CE+T)

20 (③ 120, 123 280 bp
④ 122, 121 309 bp)

25 1) 120, 121 goTaq + x7
2) 120, 121 x7
3) 124, 125

In Fusion

- 30 1) 3+4 (CERK1)
2) 5+4 (CERK1-STOP)
3) 7+4 (CERK1-trunc)

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23.9

Make more plates?

1) yeast miniprep →

E. coli transform

2) plate assay for E2A, D2A, P2A

3) goldengate. put CERK1 w/ SDMs to 3SS vector
cyto estradiol

4) leaf disc from VIGS plants → Ros tomorrow

13

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24.9 8:30 AM - 9:00 AM

P.S.

① Miniprep. 355-GERK1... → digestion → Agro trans test

② E. coli transform A9-A51-15P 25 μl not worked today

missed 1238 + 1472 in 1540 bp. significant?

missed 368 & 2161 in 1540 bp. significant?

A1 truncation at K345, K495R

A2 451 Q → *

A4 364 K → * , E575E

A13 415 R → G , 521 A → T

A14 337 Y → C , 581 P → P

A15 532 F → S

A17 359 F → S

A19 349 K → R E2A truncation?

A22 390 Y → *

A23 377 L → L , 417 A → His , 439 His → R

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quality bad

Q0 wrong primer?

October 8th. inf wed, Friday

- EST \leftarrow WT \rightarrow D44IV (FB) pick new colonies?
- 3SS \leftarrow WT? \rightarrow D44IV, F, mats
- PROCRK \rightarrow cloning to put cyto in

yeast:

D25A BleR — redo cloning \rightarrow T4 PNK IF run PCR overnight

Repeat E2A, O2A, P2A. } \rightarrow Kian.

cub — confirm cleavage. }

flow cytometry pGAL1-gmukG — SZ.

plate assay for split-ubiquitin?

re-test mutations from DE

P

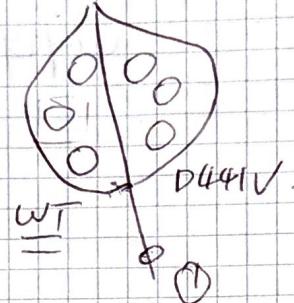
29.9.2013

b25A

- S7 check sequencing results \rightarrow miniprep + 3 tubes for S7
(... Bg9562)



- Infiltration - WT Nb.



Sequencing

T7 Standard primer

- cloning

CERK1 cyto \rightarrow SDS

SDMs



3SS

Est.

ProCEPK1

Run a gel

(0.8 ml)

Song vector

Ful insert

0.5 ml Esp3I

0.5 ml BsaI

0.5 ml T4 ligase

1 ml toxT4 buffer

10 µl intotaf

- Estradiol

brush on every infiltrated leaves

Spec.

Take leaf discs tomorrow morning



and run ROS in the afternoon

5 µl + 15 µl
(m) IPTG x-gal



c=2x1

3

30.9.2023 ~ 1.10.2023 before the report

Take leaf discs in the morning →

run ROS in the afternoon.

(use the machine
carefully, switch off
after use)

355.

Check Nb everyday, especially on Sunday
evening

Check yeast plates

reverse,
split ubiquitin

after the report.

transform. Agnos.

& confirm
regenerate mutations found in RY2H

	10 ⁺	6	2
D441V → 1.5	12	6	2
WT → 1.4	11.2	5.6	1.6
V336M → 2.5	20	10	6
F480L → 2.25	18	9	5
V336M2 → 2.5	20	10	6
V488L → 1.1	8.8	4.4	0.4
L462S → 2.0	16	8	4
M483R → 1.6	12.8	6.4	2.4
P19del → 3.8	30.4	10.1 ($\frac{1}{3}$)	6.1
P19new → 0.5G	—	—	—

$$\frac{Y}{0.5} = (L+R)$$

PCANT	1	2	3	4	5
1	control	D441V+WT	V336M+F480L	V336M2+V488L	L462S+M483R
2					
3					
4					
5					