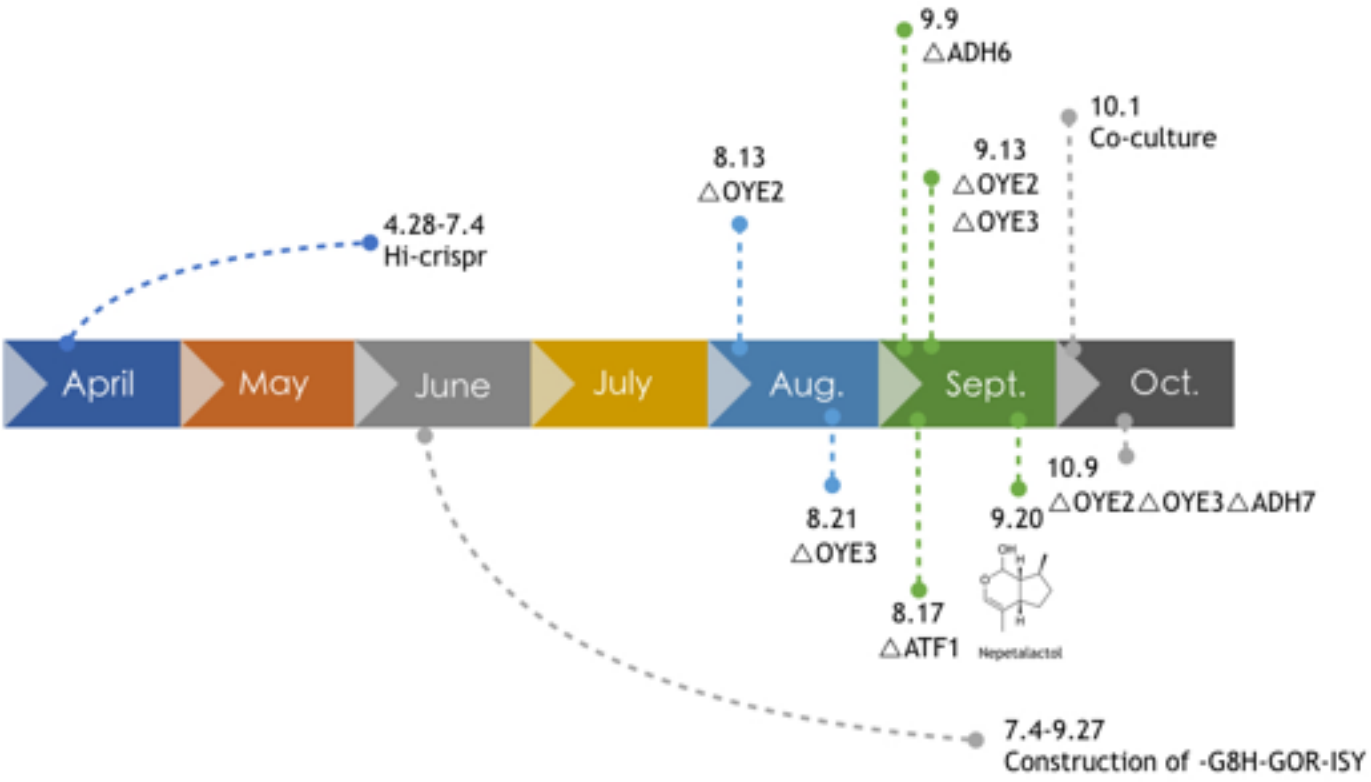
Project: Nepetalactol Synthesis

Team: GreatBay_China

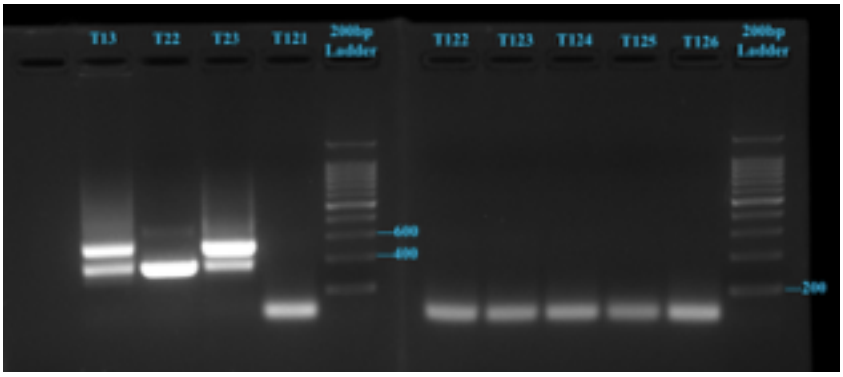
Date: 2018-4-28 to 2018-10-15



SATURDAY, 2018-4-28

1. Gradient SOE-PCR of T1O2&Regular SOE-PCR of T1O3/T2O2/T2O3

The PCR result is the following:



T1O3, T2O2, and T2O3 have correct bands, though T1O3 with another bands showing clearness.

All samples of gradient PCR of T2O2 had failed, with only primer-dimer showing clearly.

2. Assembly of pCRCT-T1O3/T2O2/T2O3

We followed the Ligation protocol, the PCR program was set as standard.

T4 Ligation Mix	15 μL
10x Ligase buffer	1.5 μL
Vector(pCRCT)	4 μL
Insertion(T_1O_3/T2O3/T2O2)	7 μL / 4.5 μL / 3 μL
T4 Ligase	1 μL
ddH2O	up to 15 μL

As insertion of T1O2 is not obtained, it is not involved in the Ligation.

MONDAY, 2018-5-28

1. **BsaI-Golden Gate assembly of: pCRCT+T1O3/T2O2/T2O3 followed by transformation**

   We repeated the pre-experiment on 28, April. pCRCT-T1O2 was failed to be assembled in last trial, so we used GenScript's gene synthesis service.

   Golden Gate assembly and Transformation process sticks to protocol.

2. **Preparation of Amp Agar dish**

   Agar plates with ampicillin were prepared according to the agar plate protocol, using pre-existing Luria-Bertinani medium.

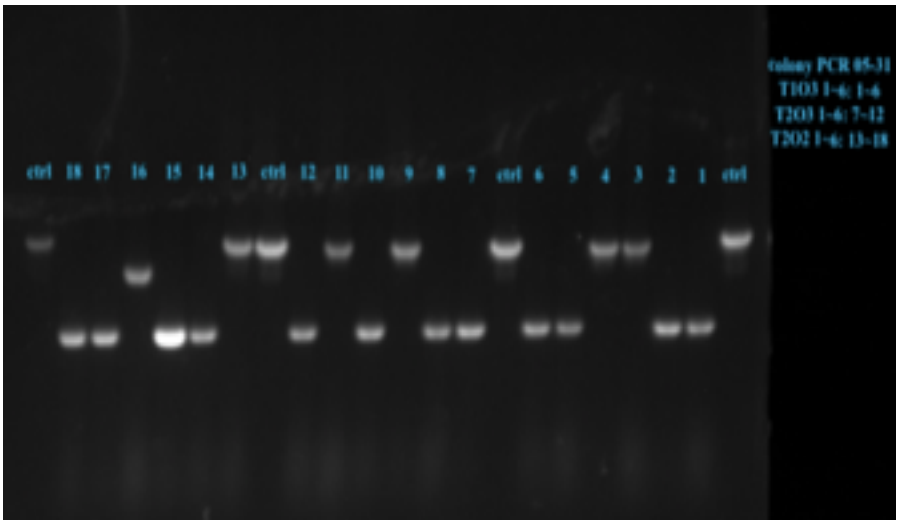
THURSDAY, 2018-5-31

1. **Colony PCR of pCRCT-T1O3/T2O3/T1O3**

   For each plate, we chose 6 colonies and made an inoculation on YPD medium. But we did not use any ladder in the gel electrophoresis due to our carelessness, so our judgement depends on the control solely.

   The PCR system follows below:

rTaq Mix	10 μL
2x master mix	5 μL
Primer F	0.5 μL
Primer R	0.5 μL
template	1 μL
ddH2O	up to 15 μL



T1O3 (3,4),T2O3 (9,11), T2O3 (16) are correct bands in comparison to the control.

FRIDAY, 2018-6-1

1. Colony PCR of pCRCT-T1O3/T2O3/T1O3

Basically, we repeated yesterday's experiment, but this time we have ladder added. The result proved yesterday's speculation is correct. T1O3(3,4),T2O3(9,11),T2O2(16) are desirable bands. We inoculated correct colonies in LB broth with Ampicillin.

TUESDAY, 2018-6-5

1. **Plasmid extraction of T1O3(3,4),T2O3(9,11),T2O2(16).**

   Process sticks to Protocol—Plasmid extraction. Preparing for sequencing.

WEDNESDAY, 2018-6-6

1. **Preparation of SC-Ura culture dish.**

   SC-Ura medium was prepared with agar concentration of 1.5%.

THURSDAY, 2018-6-7

1. **Colony PCR of BY4741：to find out the optimal method of colony PCR on Saccharomyces cerevisiae**

   OYE2&3, ADH7, and ATF1 are all knocking out sequence of yeast genome. We attempted to amplified them using primers we designed in order to verify the validity of BY4741 that we just received before the knocking out process, however appeared no brands. We therefore concluded that as the result of a false Yeast PCR method, since we changed no condition from E.coli PCR, not contemplating the presence of cell wall in yeast.

2. **Transformation of pCRCT-T1O3/T2O3/T2O2 into BY4741 strain**

   Testing of OD value to obtain growth curve of BY4741, optimum OD if transformation is 0.4. We inoculated 2 colonies as overnight pre-culture. The rest process sticks to protocol.

   Transformed BY4741 is plated on 6 SC-Ura dishes, each plasmid for 2 dishes.

Time	0h	2h	3h	4h	5h	7h
BY4141-1	0.109	0.114	0.132	0.161	0.192	0.35
BY4141-2	0.112	0.120	0.144	0.167	0.217	0.37

Time	0h	2h	3h	4h	5h	7h
Control(YPD)						

3. **Genome extraction of BY4741**

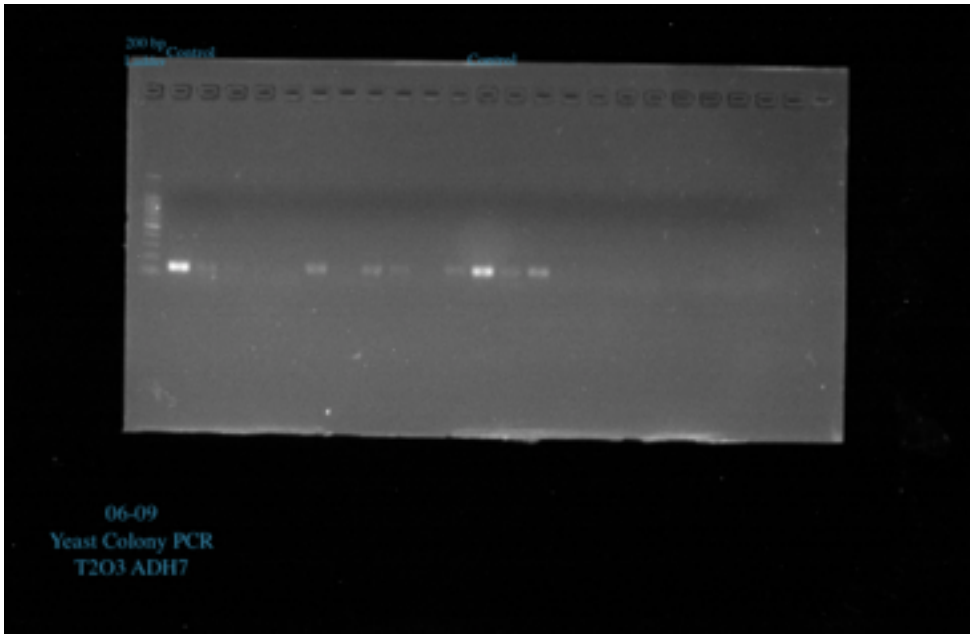
   Method of yeast genome extraction refers to GBC protocol.

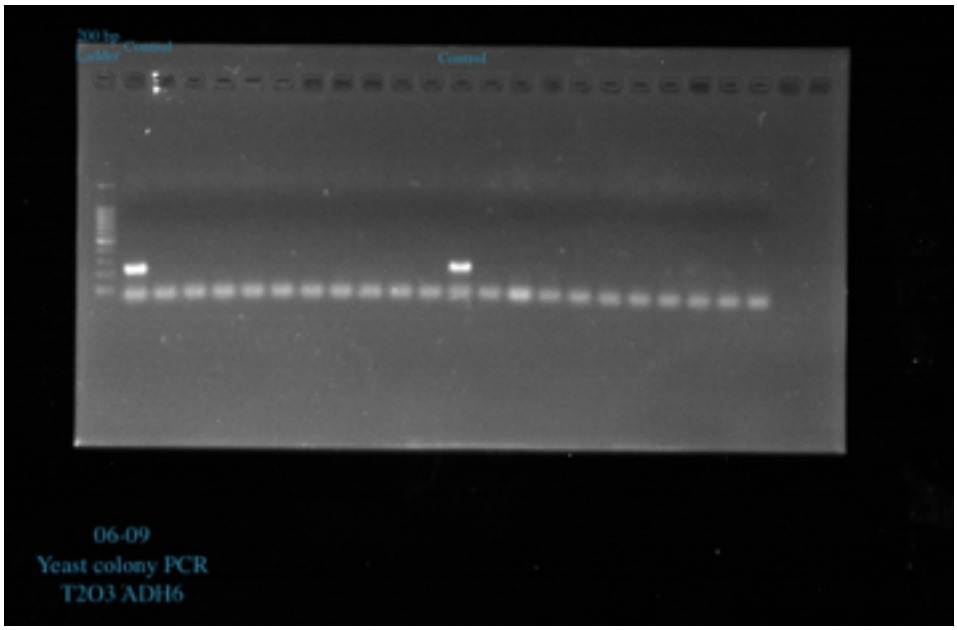
4. **Preparation of SC-Ura dishes**

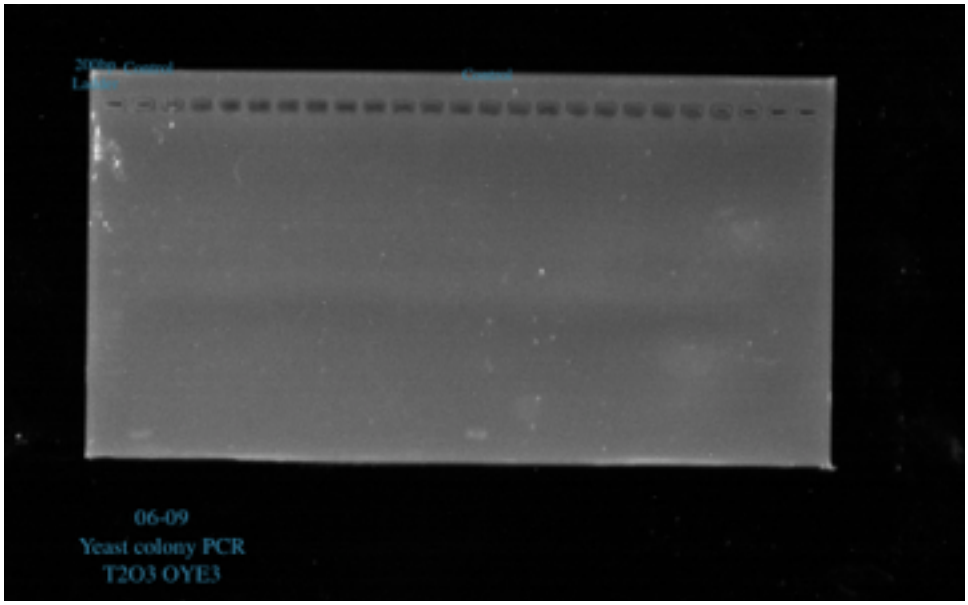
SATURDAY, 2018-6-9

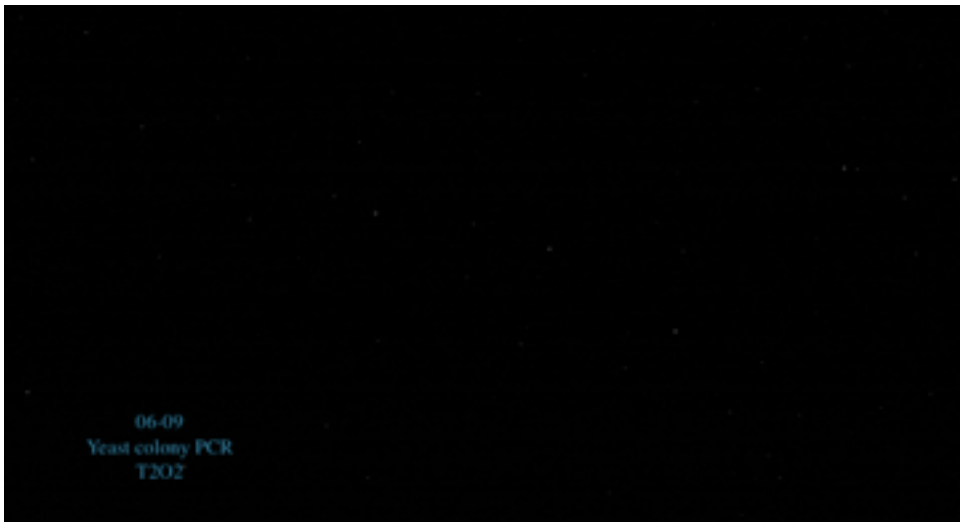
1. **Colony PCR of BY4741-pCRCT-T1O3/T2O3/T2O2**

   All dishes, in total 6, were covered with wildly growing yeast after one night's growing in 30 centigrade incubator. We thus picked 10 colonies from each dish, this is to say we have 20 colonies for one plasmid. Adding up controls we have 170 samples in total. This is the first we tried Yeast PCR in such a scale, yet we decided to break the cell walls using H2O, 98 centigrade, 10 minutes. The genome of BY4741 is used as control.




06-09
Yeast colony PCR
T2O3 ADH6


06-09
Yeast colony PCR
T2O3 OYE3


06-09
Yeast colony PCR
T2O2

Correct bands appear in none of the 4 gels. Only primer dimers and control were clearly displayed in the image. Figure 3 has vague bands presence in the bottom of the gel, indicating either high voltage or long time of electrophoresis (indeed after all the preparation it is getting late and we 'smartly' decided to step up the voltage). Later we concluded this fiasco as wrong method of breaking yeast cell wall (ideally 20% NaOH 100 centigrade 10 minutes), yet figure 4 suggests we still need to advance our IT technique.

WEDNESDAY, 2018-6-13

1. **Plasmid extraction of pCRCT-T1O2/GOR/G8H/ISY from E.coli.**

   The plasmid is obtained from Genscript Co.ltd. All plasmid extraction process stick to GBC protocol.

WEDNESDAY, 2018-6-22

1. **New BY4741 strain was plated on YPD and SC-Ura dishes. It grows on YPD and did not grow on SC-Ura. The validity of BY4741 is conformed tentatively.**
2. **New SC-Ura solid medium is prepared with gradient of Agar concentration.**

SUNDAY, 2018-6-24

1. **Retesting of the Growth Curve of the new BY4741.**

   2 colonies are inoculated. However, we did not apply sterile operation and measure OD value without removing the lid, thus, the culture might be overgrown and contaminated. We still decided to use it for next day transformation.

2. Inoculation of 2 BY4741 colonies in 5ml YPB broth, 30°C overnight as pre-culture.

MONDAY, 2018-6-25

1. Transformation of pCRCT-T1O2/T2O2/T1O3/T2O3

Overnight pre-culture was diluted 1:50 into 5ml YPD broth.

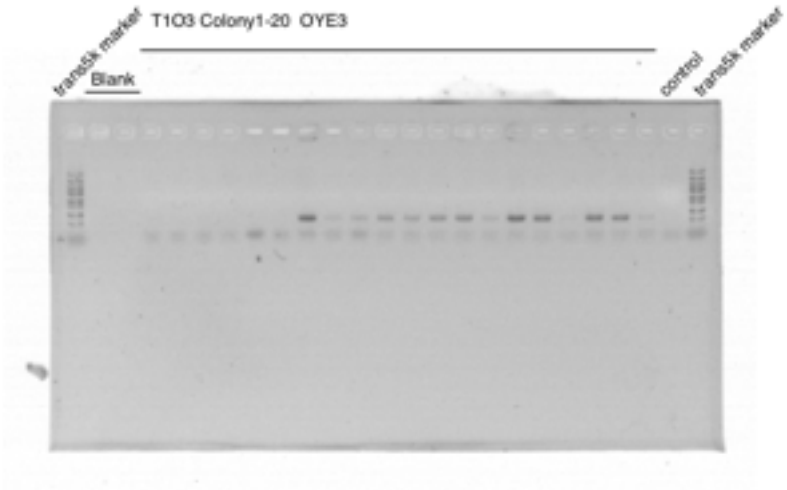
Time	0h	1h	2h	3h	4h	5h	6h	7h	8h	9h	10h
BY4141-1	0.0946	0.094	0.056	0.059	0.071	0.084	0.105	0.121	0.184	0.230	0.374
BY4141-2	0.0908	0.054	0.056	0.061	0.078	0.089	0.106	0.156	0.209	0.252	0.368
Control (YPD)	0.0901										

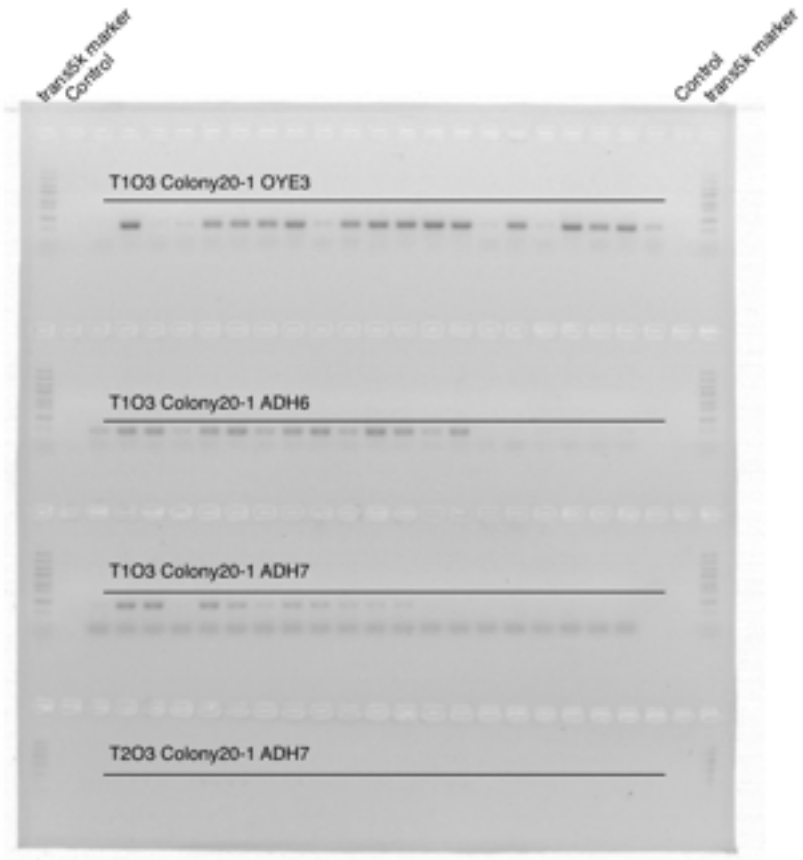
Transformation follows protocol. 100 µl cells were plated on SC-Ura dishes.(2% glucose was added as carbon source.)

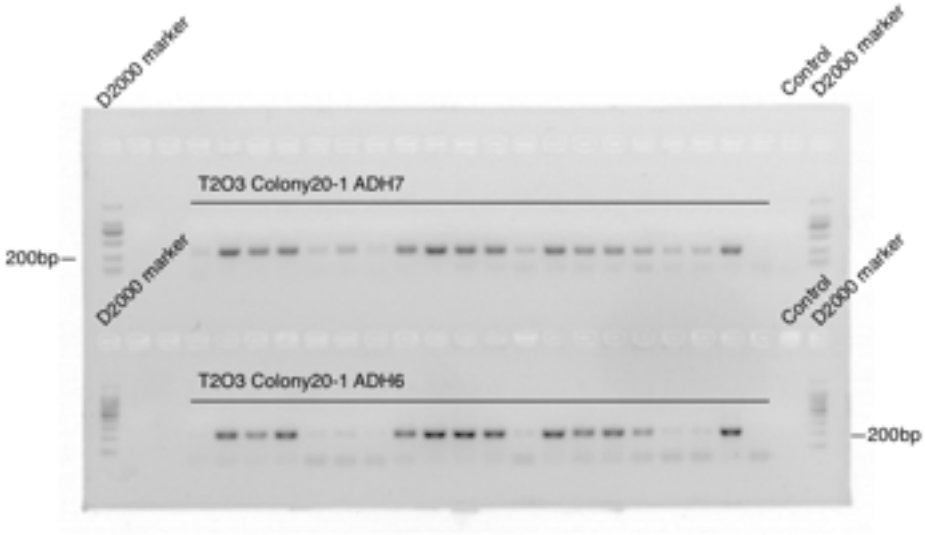
SUNDAY, 2018-7-1

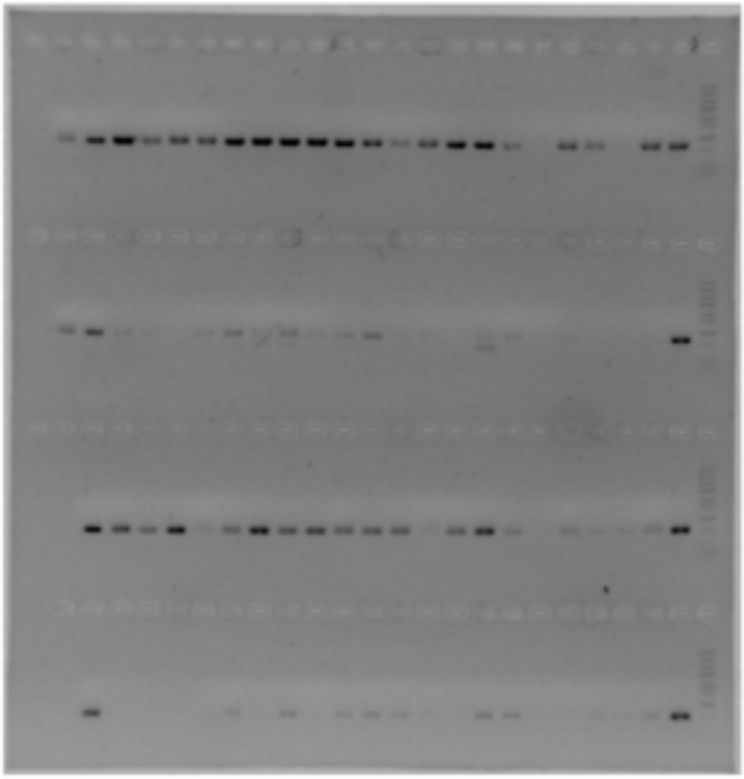
1. Colony PCR of pCRCT-T1O3, pCRCT-T2O3, pCRCT-T1O2, pCRCT-T2O2.

We chose 20 colonies from each plate. TxO2 series of plasmid contain ATF1 and OYE2. TxO3 series of plasmid contain OYE3, ADH6 and ADH7. Thus, we carried out PCR on each gene.




trans5k marker
Control
Control
trans5k marker
T1O3 Colony20-1 OYE3
T1O3 Colony20-1 ADH6
T1O3 Colony20-1 ADH7
T2O3 Colony20-1 ADH7


D2000 marker
Control
D2000 marker
T2O3 Colony20-1 ADH7
200bp
D2000 marker
Control
D2000 marker
T2O3 Colony20-1 ADH6
200bp



20180701 Colony PCR
T1O2+T2O2
1st row marker(5k) control T2O2 ATF1 1-20 control;
2nd row marker control T1O2 ATF1 1-20 control;
3rd row marker control T2O2 OYE2 1-20 control control;
4th row marker control T1O2 OYE2 1-20 control control.

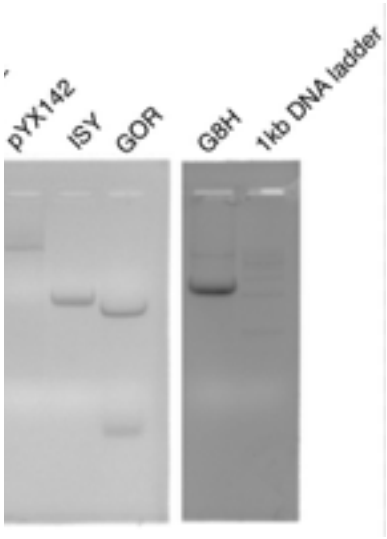
T2O3/T2O2 series of plasmid should be 100bp shorter than control, which is the cds of each gene from the genome of BY4741. Thus, we concluded that no correct results of T2Ox.

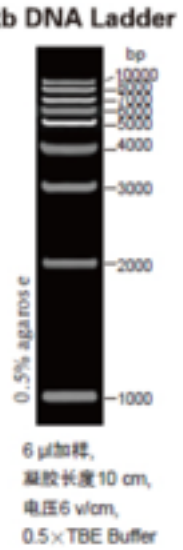
Bands of T1O3 should be same as the control as they only has the difference of 8bp. Thus, remaining PCR system of “T1O2 Colony 1 3 4 6 7 8 10 11 12 13 14 15 16 17 18 19 20” and “T1O3 1-12” were sent for sequencing.

Controls of T1O3 and T2O3 are not shown.

WEDNESDAY, 2018-7-4

1. **Construction of pYX142-G8H-GOR-ISY——First plan of nepetalactol expression pathway.**

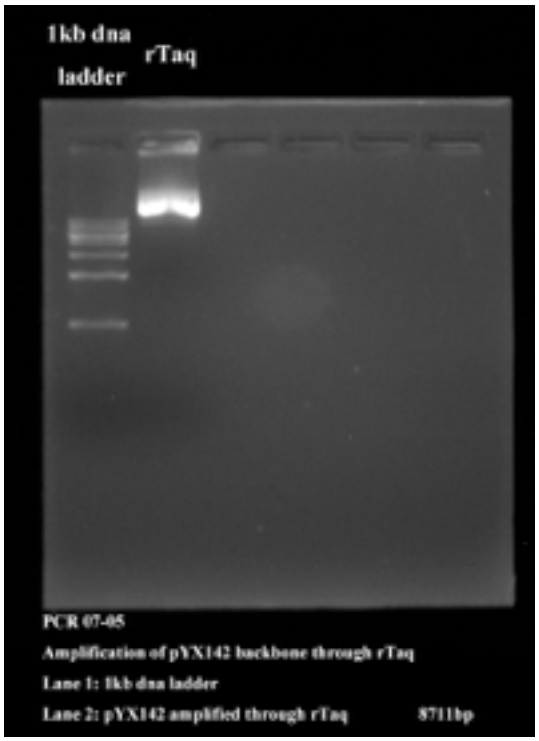


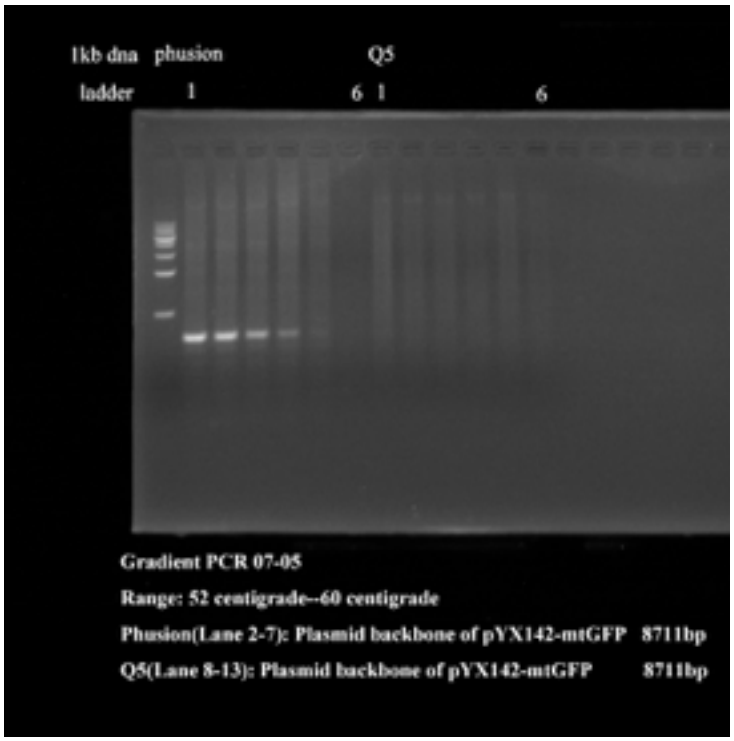


pYX142 was amplified with unspecific bands, the rest were clear and correct. ISY, GOR and G8H were performed with gel isolation and gel extraction.

THURSDAY, 2018-7-5

1. **Construction of pYX142-G8H-GOR-ISY——amplification of pYX142**





Amplified in a system of 10μl each over the range of 52°C to 60°C. 3 polymerases were used(Phusion， Q5 and rTaq). With primers pYX142F and pYX142 R.

The fragment of pYX142backbone was more than 10000bp whereas the entire plasmid is only of 8711bp. The gradient PCR performed using phusion and Q5 gives smear bands and primer-dimers.

2. Miniprep of co-culture medium.

1L of co-culture medium was prepared, ingredients listed below:

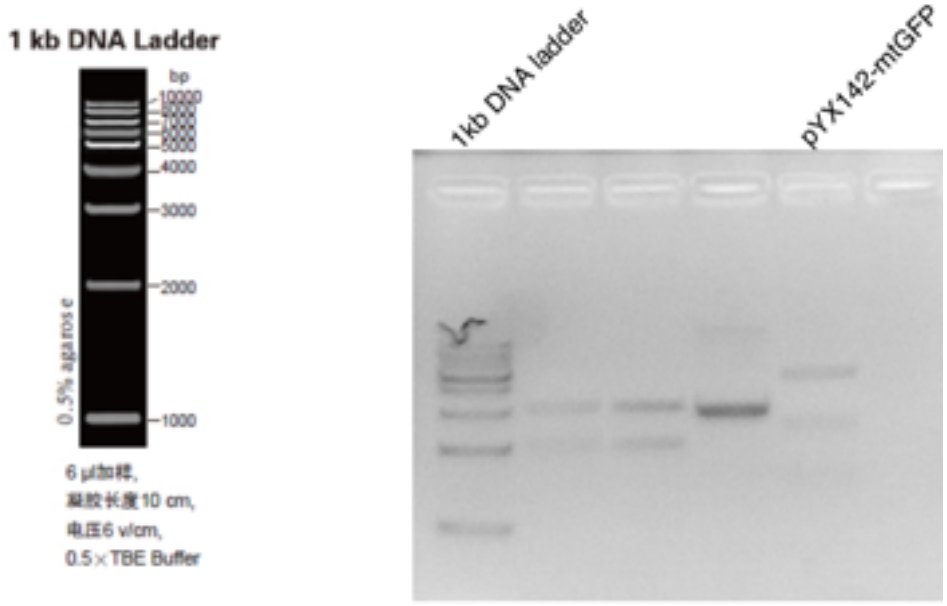
KH_2PO_4	13.3g
$(NH_4)HPO_4$	4g
yeast extract	5g
citric acid	1.7g
$MgSO_4$ $7H_2O$	1.3g
EDTA	0.0084g
$CoCl_2$	0.0025g
H_3PO_4	0.0030g
Na_2MoO_4	0.0025g
Fe(III) citrate	0.06g
thiamine	0.0045g
$Zn(CH_3COO)_2$	0.008g
$MnCl_2$	0.015g
$CuCl_2$	0.0015g

The amount of minor elements were inaccurate as we added them in the scale of 1L directly. Mother liquor should be used to enlarge the scale of minor elements. We will repeat the experiment.

FRIDAY, 2018-7-6

1. Restriction of pYX142-mtGFP.

Due to failures in amplification of pYX142 backbone, we carried out this test on pYX142-mtGFP using EcoRI. 3 fragments should be formed corresponding to the length of 1.5kb, 2.5kb and 4.7kb.



2 Bands appeared to be around 5kb and 2.5kb. The band expected to be 1.5kb is not showed and existing bands are faint. Nevertheless, the restriction result fits our expectation.

Though result of the restriction were performed as expected, we still decided to built GOR-ISY-G8H metabolic pathway on pYES as other potential causes are excluded.

THUESDAY, 2018-7-10

1. Genome extraction of T1O3-8 for sequencing.

Glycerol stock was made with 300μl culture + 300μl 50% glycerol solution.

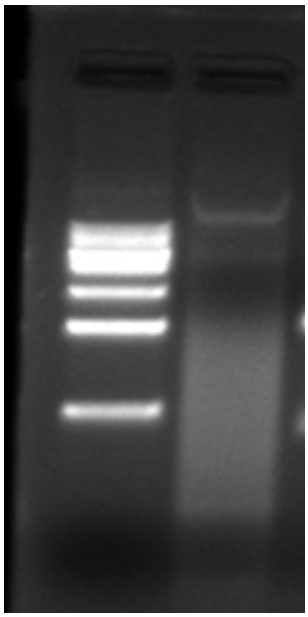
	conc. (ng/μl)
T1O3-8 genome 1	608.85
T1O3-8 genome 2	651.35

FRIDAY, 2018-7-13

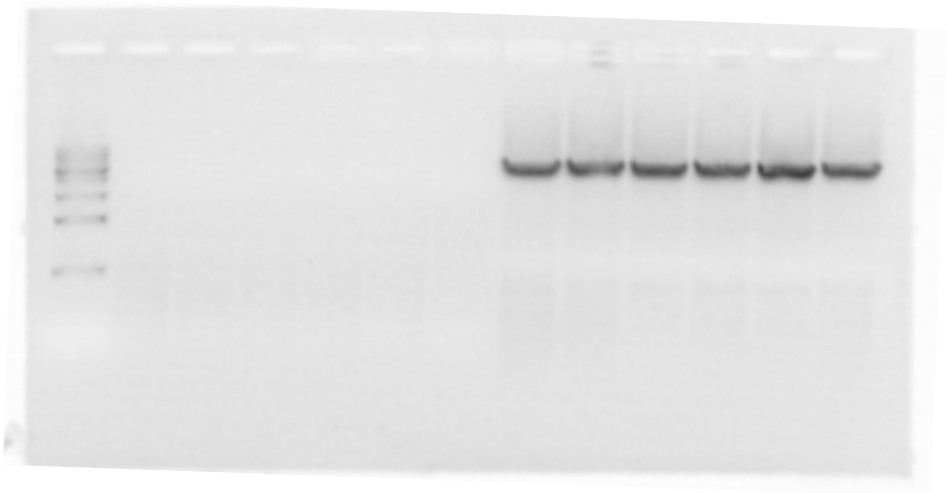
1. Construction of pYES-G8H-GOR-ISY

Amplification of fragment pYES with Primers “pYES Fnew”and ”pYES Rnew”. Phusion polymerase is used with an extension time of 2min30s.

However, MgCl2 was not added into the system as we want it to be. 2 obscure bands were seemed due to unspecific binding site. Smear bands might due to excessive template.



Amplification of pYES was repeated using same primers with rTaq master mix and Phusion polymerase. Temperature ranges from 52-60°C.



rTaq amplified 6 samples of pYES, thus we performed PCR product purification.

SATURDAY, 2018-7-14

1. Gibson assembly of pYES-ISY-G8H-GOR was performed as below:

	pYES	ISY	G8H	GOR
conc. (ng/μl)	12.6	139.8	86.65	104.65
length (bp)	5000	2170	2417	1830
Vol. (μl))	3.5/4.3	0.5 / 0.2	0.5/0.3	0.5/0.2

2 sample of gibson product each of 10μl were transferred into 100μl of DH5α competent cell.

Notation: “SG 7.5” “SGO 7.5”

SUNDAY, 2018-7-15

1. Redo the Gibson assembly of pYES-ISY-G8H-GOR

Plate from 7.14 shows no colony, might due to low conc. of plasmids.

We repeat the gibson&transformation.

	pYES	ISY	G8H	GOR
conc. (ng/μl)	12.6	139.8	86.65	104.65
length (bp)	5000	2170	2417	1830
Vol. (μl))	3.5	0.5	0.5	0.5

MONDAY, 2018-7-16

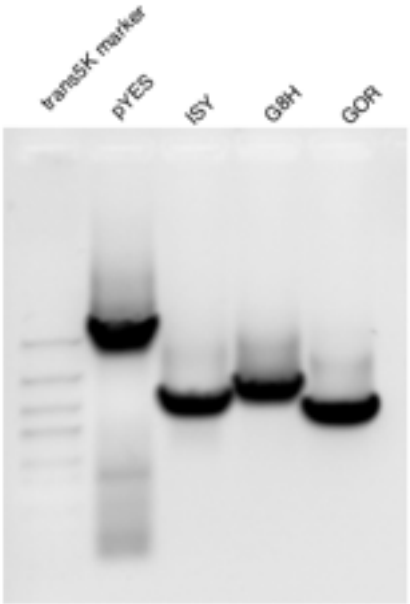
1. Gibson assembly of pYES-ISY-G8H-GOR

4 fragments were amplified again with rTaq master mix.

Gibson assembly system was carried out using 2 ratios.

FRAGMENT	LENGTH	PRIMER F	PRIMER R	TEMPLATE
pYES new	5000bp	pYES Fnew	pYES Rnew	pYES-mtGFP
ISY	2170bp	ISF F	ISY R	pSB1C3-ISY
G8H	2417bp	G8H F	G8H R	pSB1C3-G8H
GOR	1830bp	GOR F	GOR R	pSB1C3-GOR

PCR product purification	conc. (ng/μl)
pYES	36.15
ISY	83.73
G8H	73.3
GOR	111.05

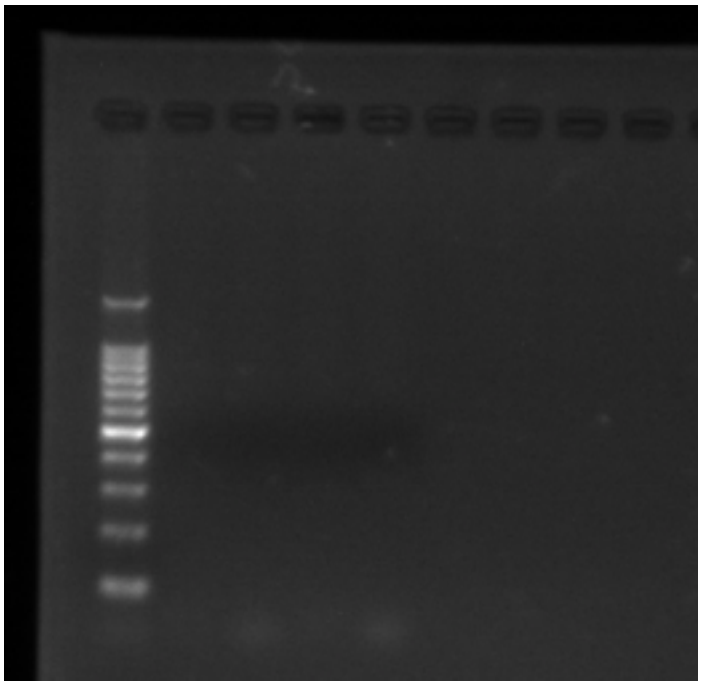


	pYES	ISY	G8H	GOR
Ratio1: Vol. (μl))	2	0.5	0.5	0.3
Ratio2: Vol. (μl))	3.2	0.8	0.4	0.6

TUESDAY, 2018-7-17

1. **Transformation of pYES-ISY-G8H-GOR into DH5α.**
2. **Colony PCR of pYES-G8H-GOR from 7.14 and 7.15**

   For each plate 2 colonies were picked. 2 pairs of primers are used: G8H VF&TEF1 VR, GOR VF&PRP VR, corresponding to 582bp and 638bp. Failed.



3. **Quality test for primer of traditional gene Knockout colony PCR**

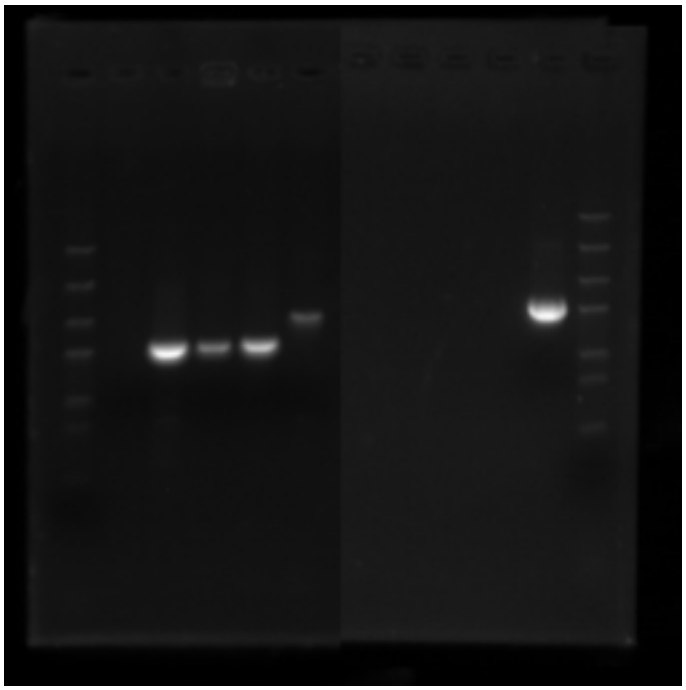
   Primers was diluted to 10x. PCR system was 10μl for each pairs of primer.

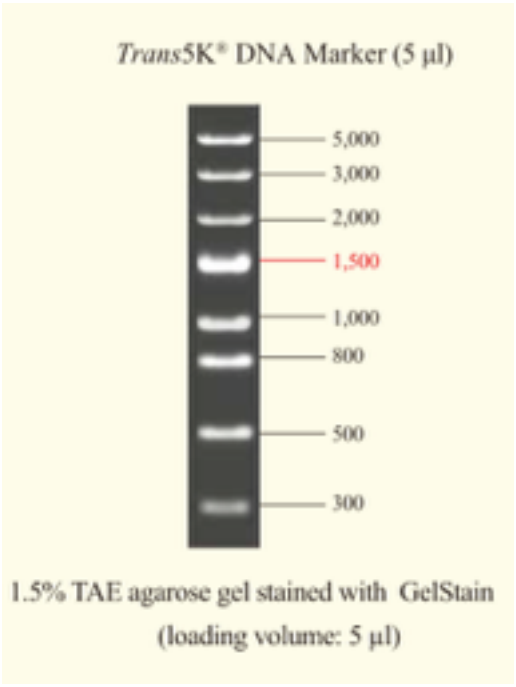
   ATF1 failed in first round, thus it was repeated.

   Result shows the validity of all primers.

FRAGMENT	LENGTH	PRIMER F	PRIMER R	TEMPLATE
ATF1	2kb	T-ATF1(VF)	T-ATF1(VR)	T1O3-8 genome

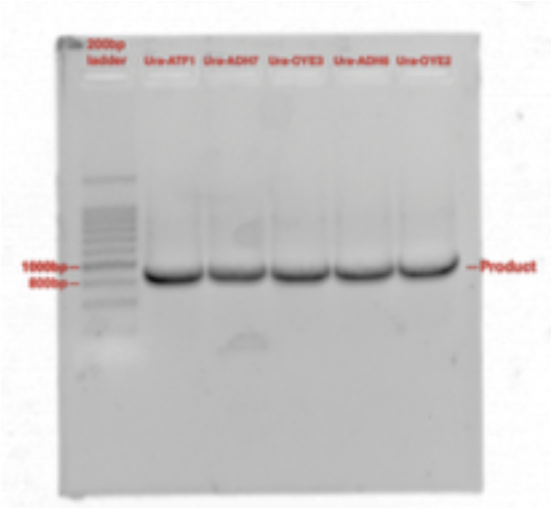
FRAGMENT	LENGTH	PRIMER F	PRIMER R	TEMPLATE
OYE2	1.5kb	T-ATF1(VF)	T-ATF1(VR)	T1O3-8 genome
OYE3	1.5kb	T-ATF1(VF)	T-ATF1(VR)	T1O3-8 genome
ADH6	1.5kb	T-ATF1(VF)	T-ATF1(VR)	T1O3-8 genome
ADH7	1.5kb	T-ATF1(VF)	T-ATF1(VR)	T1O3-8 genome





4. Amplification of homogenous arms on Ura sequence

PCR product purification	conc. (ng/µl)
Ura-ATF1	69.0
Ura-OYE2	53.5
Ura-OYE3	71.4
Ura-ADH6	78.1
Ura-ADH7	105.95



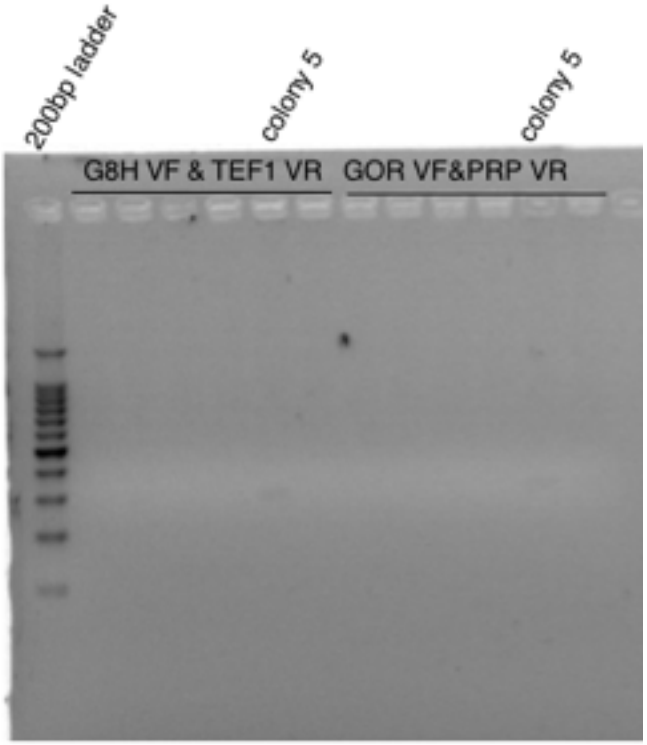
WEDNESDAY, 2018-7-18

1. Colony PCR of pYES-GOR-G8H-ISY from 7.17

6 colonies was picked from each plate, 12 colonies in total.

Same primers for colony PCR(rTaq) were used. (annealing temperature of 56°C and extension time of 45s)

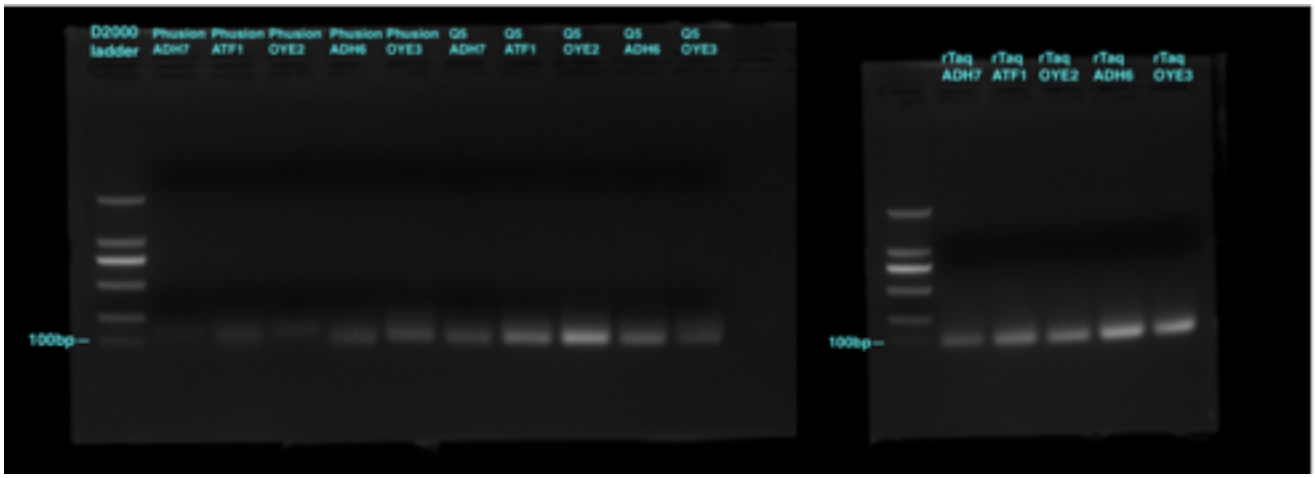
Correct bands should be 582bp and 631bp.



2 bands of colony 5 were considered as correct, though they are less obvious.

2. Amplification of homogenous arms of knockout-genes

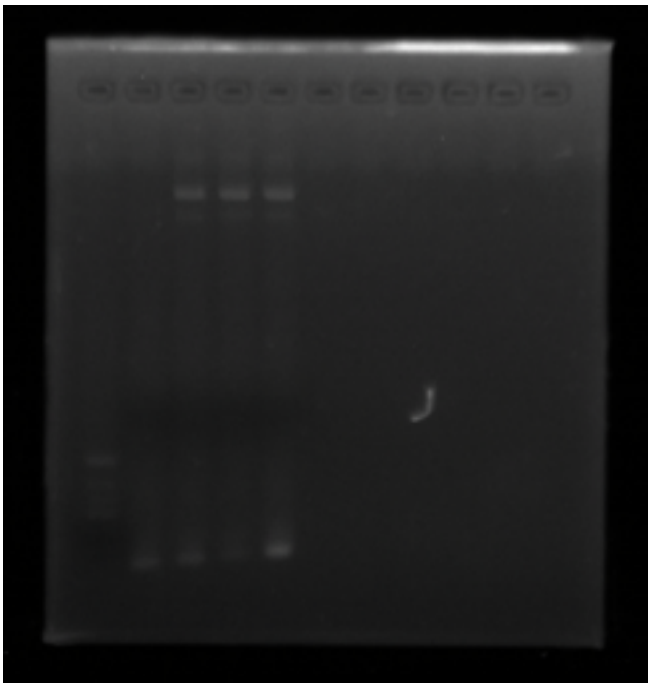
Annealing temperature of 52°C is fine.



3. Amplification of fragments of homogenous arms with sticky arms for golden gate assembly.

FRAGMENT	LENGTH	PRIMER F	PRIMER R	TEMPLATE
OYE2	152bp	OYE2ggF	OYE2gg2	pCRCT-T1O2
OYE3	156b	OYE3ggF	OYE3gg2	pCRCT-T1O3
ADH6	156b	ADH6ggF	ADH6ggR	pCRCT-T1O3
ADH7	150b	ADH7ggF	ADH7ggR	pCRCT-T1O3

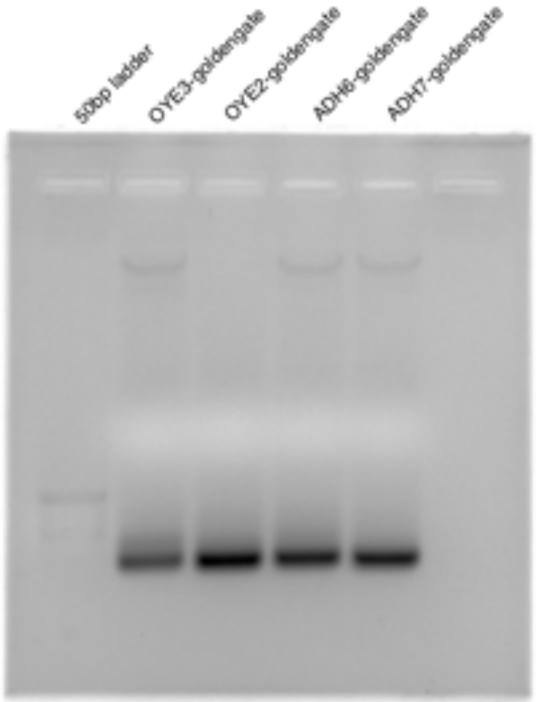
All samples shows no correct bands. This experiment will be repeated.



THURSDAY, 2018-7-19

1. Repeat the Amplification of fragments of homogenous arms with sticky arms for golden gate assembly.

Same primers were used as yesterday, with a rTaq system of 50μl. Annealing temperature was adjusted to 57°C and extension time of 30s.



2. Preparation of co-culture medium

The following were directly added to the main culture.

KH_2PO_4	13.3g
$(NH_4)HPO_4$	4g
yeast extract	5g
citric acid	1.7g
$MgSO_4$ $7H_2O$	1.3g

1000x Mother liquors were prepared in 4 portions.

1. Trace mineral
2. Vitamin B
3. EDTA
4. ferric ammonium citrate

Heat Sterilization Treatment by autoclave.

FRIDAY, 2018-7-20

1. Transformation of Ura-ha of OYE2, OYE3, ADH6, ADH7 and ATF1

Due to unavailability of plate reader, we estimated the OD value.

Notation:”7.20 OYE2” ”7.20 OYE3” ”7.20 ADH6” ”7.20 ADH7” ”7.20 ATF1”

Plated on SC-Ura dishes using glass beads.

2. Construction of pCRCT-OYE2/OYE3/ADH6/ADH7 using golden gate assembly

Conc. of PCR product purification were:

OYE2: 10.3 ng/μl OYE3: 11.75ng/μl ADH6:17.7ng/μl ADH7: 11.0ng/μl

pCRCT plasmid was diluted 1:10, by adding 5μl of plasmid(292.3ng/μl) into 45μl ddH2O.

Golden gate	20 μL
T4 ligase buffer	2 μL
T4 DNA ligase	1 μL
BsaI-HF	1 μL
pCRCT	3 μL
OYE2/OYE3/ADH6/ ADH7	1 μL
ddH2O	12 μL

setting：

37°C	5’
16°C	10’
37°C	15’ **10repeats**
50°C	5’
80°C	5’
12°C	forever

Golden gate products were transformed into DH5α and plated on LB dishes.

SATURDAY, 2018-7-21

1. re-construction of pCRCT-OYE2/OYE3/ADH6/ADH7 using golden gate assembly

Golden gate system follows…..

Transformed into 50μl DH5α, original pCRCT plasmid was transformed as positive control.

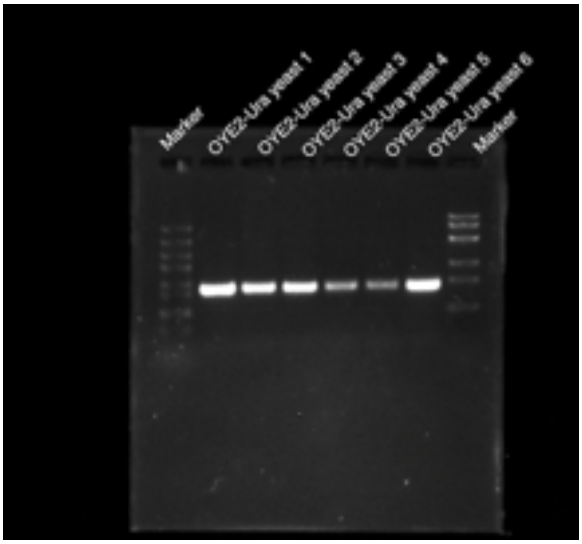
Plated on Amp LB dishes and Placed in 37°C for overnight growth.

SUNDAY, 2018-7-22

1. re-construction of pCRCT-OYE2/OYE3/ADH6/ADH7 using golden gate assembly

No colonies has grown on the plates of 7.21. Thus, golden gate was repeated and transformation was carried out more cautious. 100μl chemically competent DH5α was used.

2. Colony PCR of BY4741 containing Ura-OYE2ha



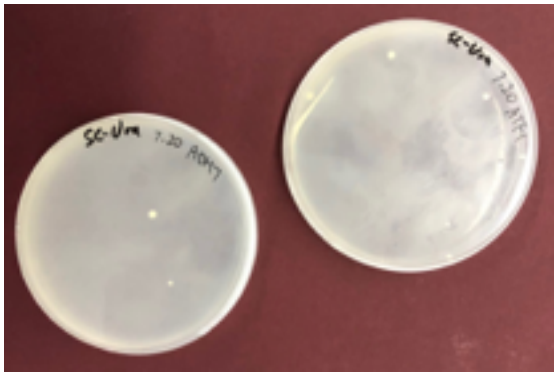
Length of the bands met expectation, thus 6 colonies was inoculated into 5ml YPD with 2% glucose as pre-culture.

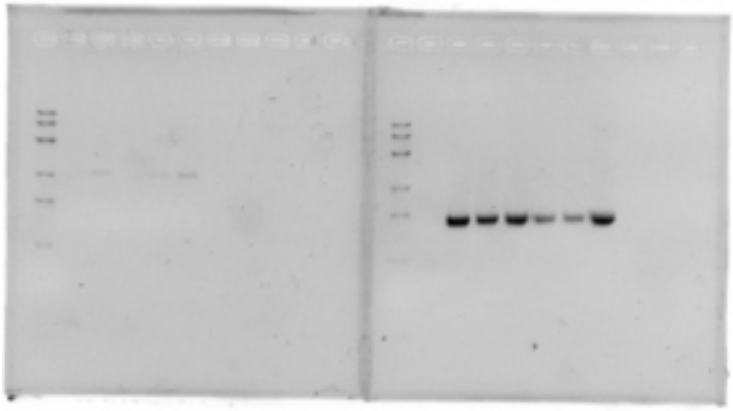
3. 1L of YPD broth and 1L of 20% glucose was made and sterilized.

MONDAY, 2018-7-23

1. Colony PCR of Traditional knockout

Control uses the same colony PCR primers, but the template is BY4741 genome. Also, ADH7 and ATF1 had colonies grown.





20180723 4741 genome PCR Trans8K OYE2 OYE3 ATF1 ADH6 ADH7

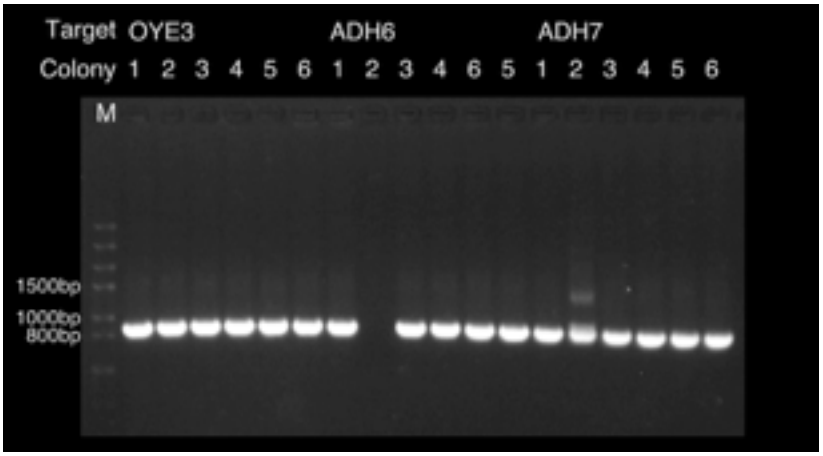
Trans8K OYE2control 1~6.Tif

All 6 colonies show correct bands.

2. Colony PCR of DH5α containing pCRCT-OYE3/ADH6/ADH7

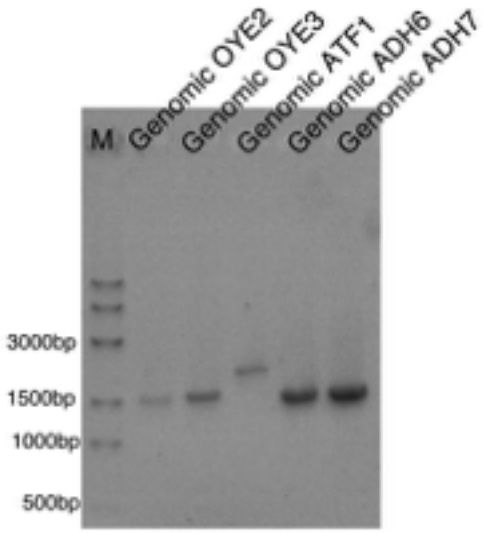
As traditional knockout has achieve some progression, pCRCT-OYE2 was not checked by colony PCR.

All colonies had shown correct bands except pCRCT-ADH6#6.



Remaining PCR system was sent for sequencing.

3. Amplification of genomic negative control.



Gel extraction was carried and purified controls are stored in 4°C freezer.

TUESDAY, 2018-7-24

1. Prep. for plasmid extraction of pCRCT-ADH6/ADH7/OYE3

Inoculation of colonies from replicate plate into 5ml amp LB broth.

SATURDAY, 2018-7-28

1. Transformation of homogeneous arms into BY4741-△OYE2-containing Ura

The growth rate of BY4741 decreased a lot to reach stationary phase, thus we thought the pre-culture did not grow on last few days' attempt.

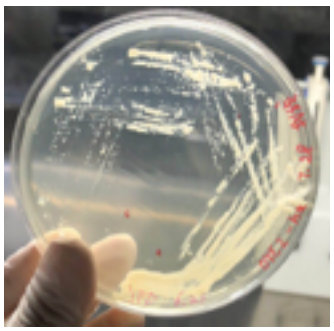
6 tubes of Pre-culture were diluted 1:100 into 30ml YPD

after 13h, OYE2－ha（100bp linear DNA）was transformed into BY4741-△OYE2-containing Ura.

Plated on YPD and grew at 30°C

TUESDAY, 2018-7-31

1. Streak lawn plate of 7.28 on YPD



Competent cells were not diluted before plating on YPD.

2. Overnight culture of pYES-G8H-GOR-ISY

6 colonies were inoculated. shaken for 19hours

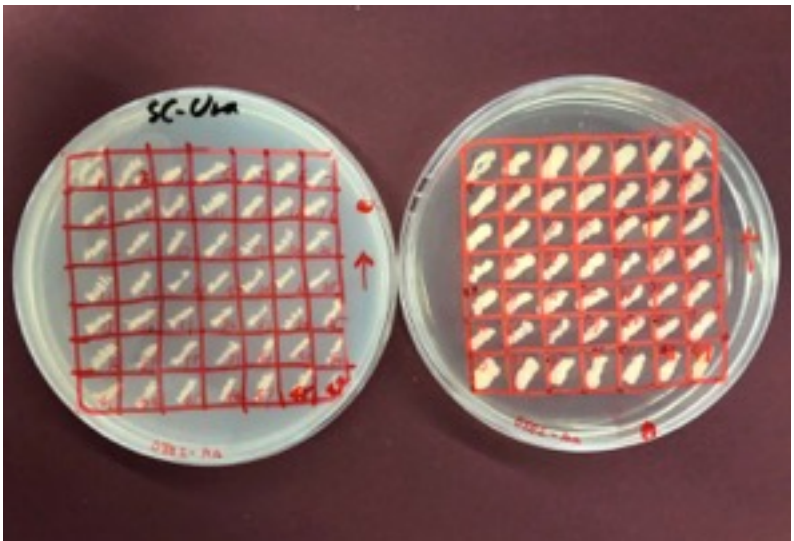
3. Hi-crispr multigene knockout in BY4741

BY4741 streaked on YPD medium.

WEDNESDAY, 2018-8-1

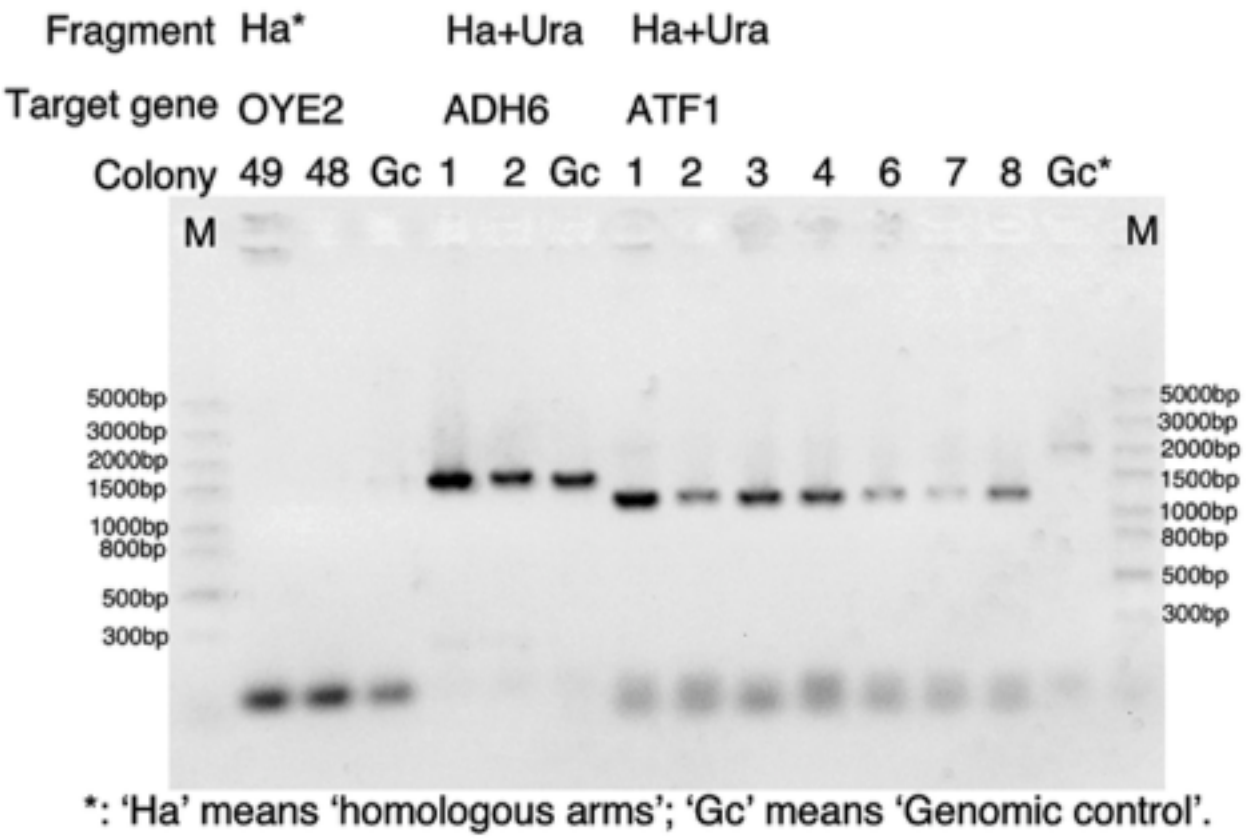
1. Colony screening of BY4741-△OYE2

Free colonies on the streaked plate were picked and replicated on SC-Ura plate first then YPD plate.



Colony # 49 seemed to be inactivated on the SC-Ura plate. #49 will be further tested by culturing in liquid SC-Ura.

2. Colony PCR of BY4741 containing Ura-ha of OYE2/ADH6/ATF1



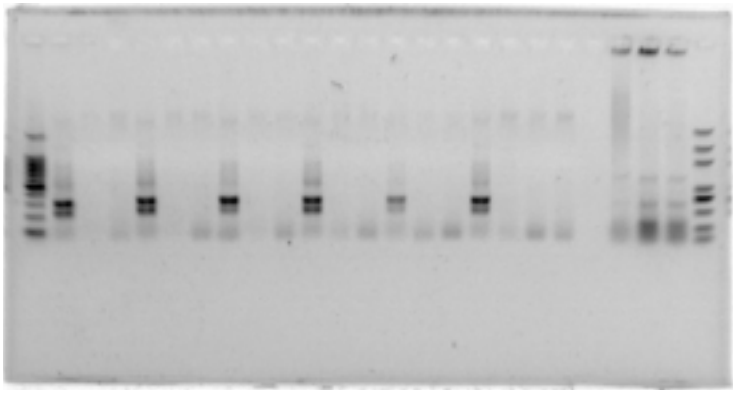
OYE2 might not replace Ura with the homogeneous arm. All ADH6 have incorrect bands and ATF1 all has correct bands.

BY4741-△ATF1 was obtained.

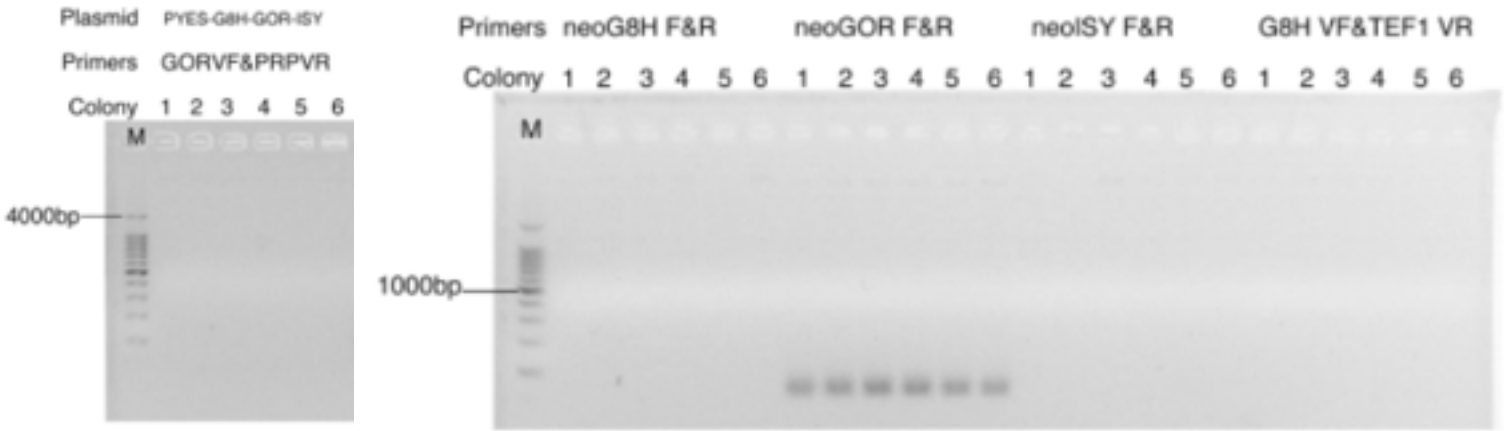
3. Plasmid extraction of pYES-G8H-GOR

For sequencing and for PCR checking.

4. Plasmid PCR



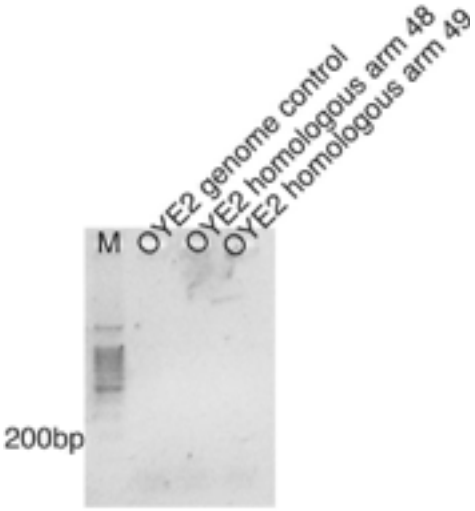
pYES-G8H-ISY-GOR was identified as failure in construction, but this assay will be repeated.



THURSDAY, 2018-8-2

1. **Plasmid extraction of pCRCT-OYE3/ADH6/ADH7**
2. **Amplification of OYE2-ha, ATF1-Ura**
3. **Colony PCR of BY4741-△OYE2 containing homogeneous arm of OYE2**

   We adjusted the extension time from 30s to 90s. Colony #49 & #48 were picked.



Improvements：

- Use primers of ATF1 as control
- Use OYE2-ha-F1/R1 as Primers of colony PCR
- Use another genome of BY4741
- Increase vol of template to 2μl

FRIDAY, 2018-8-3

1. Overnight culture of BY4741, BY4741-△OYE2 with Ura, BY4741-△ATF1 with Ura

Inoculated into 3ml YPD with 2% of glucose.

2. PCR amplification of ATF1-ha. PCR product purification of OYE2-ha and ATF1-Ura.

2. SC-Ura solid culture preparation

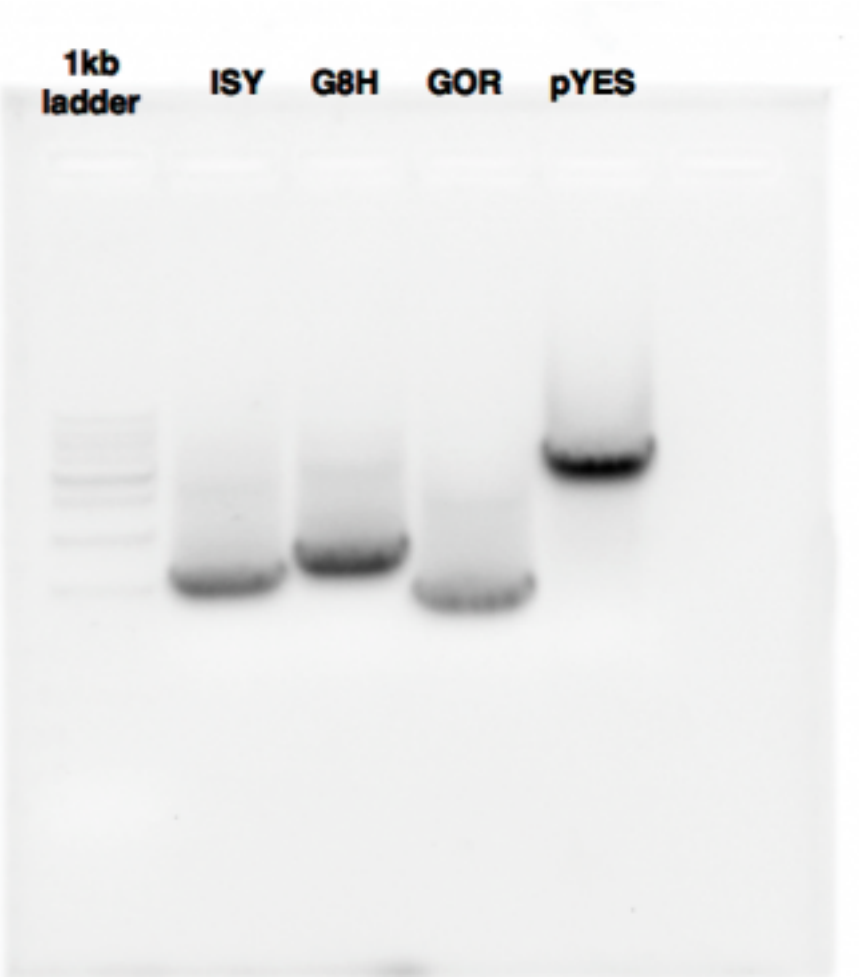
1L of SC-Ura containing 5% of Agar was made and sterilized

SATURDAY, 2018-8-4

1. Re-construction of pYES-ISY-GOR-G8H

Amplification of pYES, ISY, GOR, G8H fragments

Gel extraction and gibson assembly was performed.



2. Transformation

pCRCT-OYE3/ADH6/ADH7 into Strain BY4741，Plated on SC-Ura

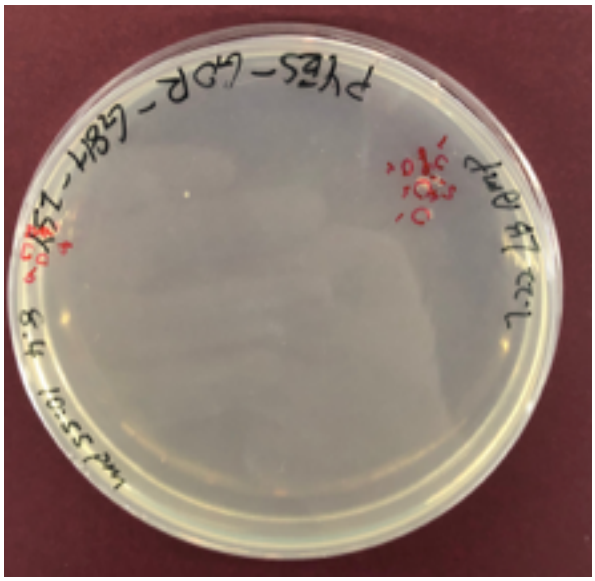
ATF1-ha into Strain BY4741-△ATF1 with Ura, diluted and plated on YPD

OYE2-ha into Strain BY4741-△OYE2 with Ura, diluted and plated on YPD

pCRCT-T1O2/T1O3/T2O2/T2O3 into BY4741, plated on SC-Ura.

SATURDAY, 2018-8-6

1. Colony PCR of DH5α containing pYES-GOR-G8H-ISY



9 colonies were picked from the plate. Later we suspected there were 2 real colonies and the rest were satellite colonies as the plate was cultured for 2 days. G8H VF/TEF1 VF and GOR VF/PRP VR were used and each colony was checked by 2 pairs of primers. rTaq system follows protocol. Colony were diluted in 5μl ddH2O for making replicate and 2 PCR systems. Failed with no bands.

2. Preparation of SC-Ura solid medium

20μl NaOH solution was added each time untill pH reached 5.5

2% agar

SUNDAY, 2018-8-7

1. Checking the design of pYES-ISY-GOR-G8H

2. Colony PCR of BY4741/OYE2-ha

replicate on both YPD and SC-Ura plate.

Although 5 colonies all had correct band of 100bp, the control also shows the band of same length. Thus the PCR result is not raliable.

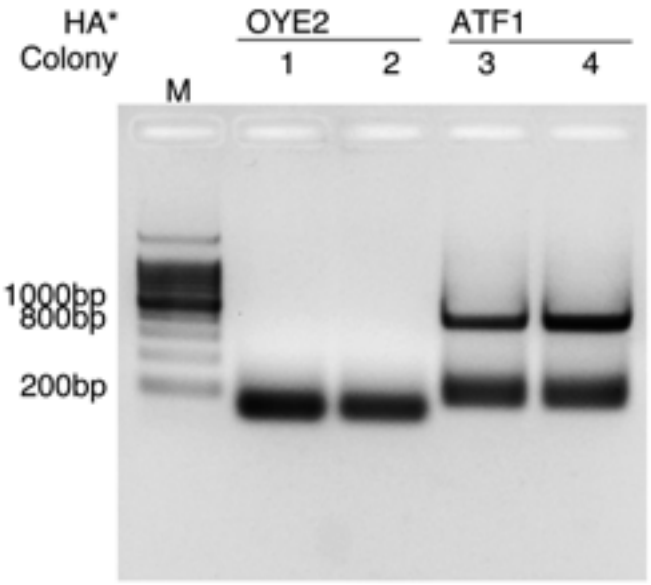
3. Primer testing of colony PCR primers desinged for pYES-ISY-GOR-G8H

neoG8HVF/VR(1857bp), neoGORVF/VR(1277bp), neoISYVF/VR(1396bp)

all clear and bright band shown.

pSB1C3-ISY/GOR/G8H were used as template.

3. Amplification of OYE2ha and ATF1 ha.



*'HA' means the homologous arms of a fragment

SUNDAY, 2018-8-8

1. **Redo the colony PCR of DH5α/pYES-ISY-GOR-G8H using neoG8HVF/VR(1857bp), neoGORVF/VR(1277bp), neoISYVF/VR(1396bp)**

   Only #3 4 5 6 9 grew on the replicate plate.

No bands appeared on the gel image.

2. Transformation

ATF1-ha into Strain BY4741△ATF1 with Ura, diluted and plated on YPD

OYE2-ha into Strain BY4741△OYE2 with Ura, diluted and plated on YPD

Placed in 30°C incubator.

3. Colony PCR of pCRCT-TnOn series transformed from the date of 7.4

6 colonie were picked for each plasmid.

For TnO2, ATF1 VF/VR and OYE2 VF/VR were used.

For TnO3, OYE2 VF/VR, ADH6 VF/VR and ADH7 VF/VR were used.

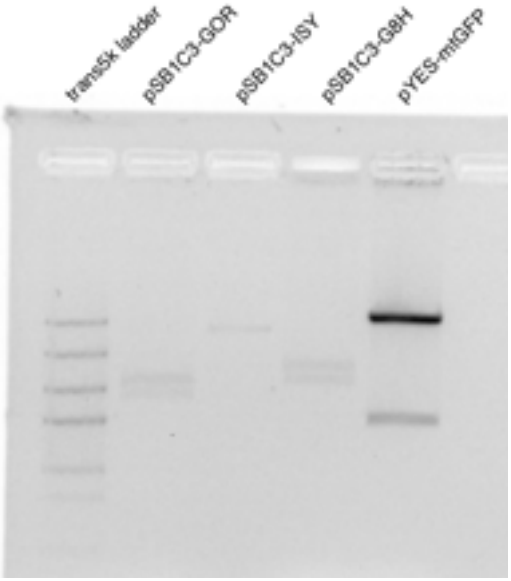
缺result

4. Construction of pYES-ISY-G8H-GOR plan 2——RD and ligation

RD of pSC1C3-ISY using EcoRI and XbaI; RD of pSB1C3-GOR using EcoRI and SpeI

RD of pSB1C3-G8H using SpeI and EcoRI; pYES-mtGFP using EcoRI and SpeI

50μl RD system follows protocol.



All shows correct bands, gel extraction was performed.

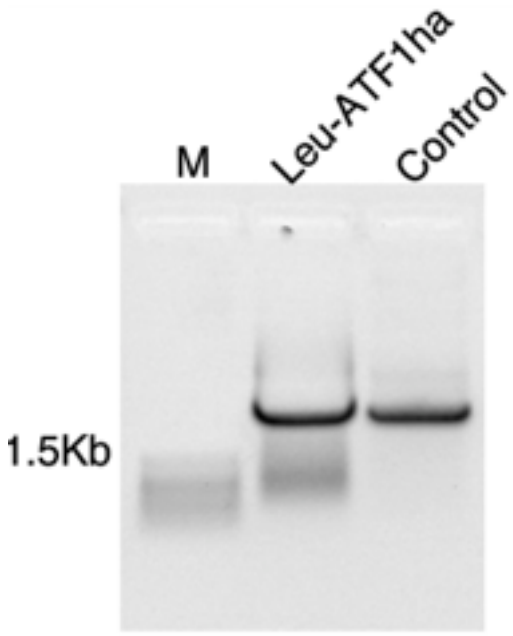
Ligation was carried out to construct pSB1C3-ISY-GOR, pYES-G8H. Left on the Thermomixer for 10h40min.

MONDAY, 2018-8-9

1. Inoculation of BY4741△OYE2/OYE2ha in liquid SC-Ura for re-screening.

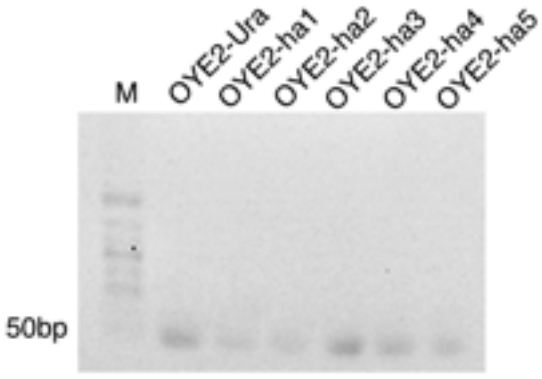
2. Amplification of LEU-ATF1

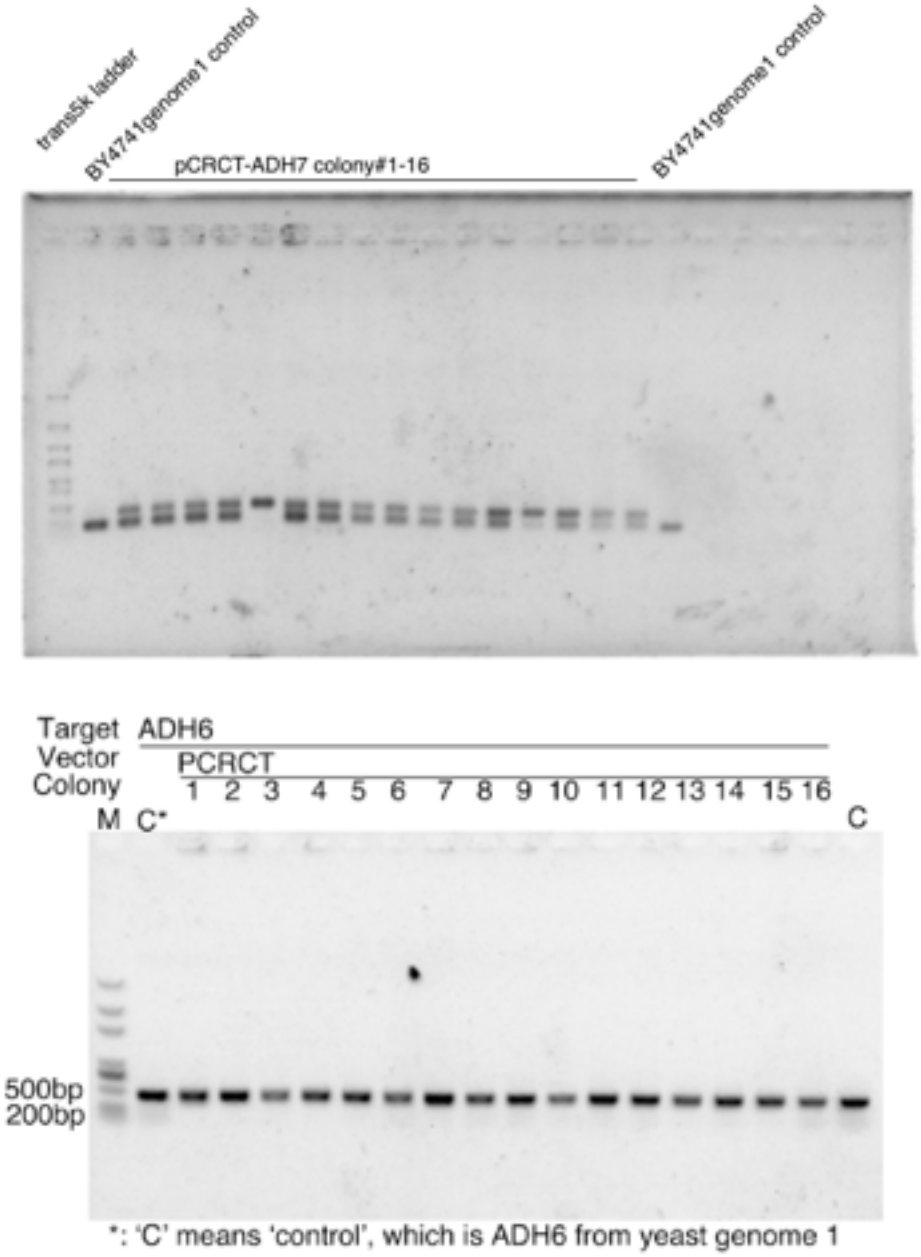
Control was the remaining system of first round amplification.



3. Colony PCR of BY4741/ OYE2ha with Ura and BY4741△OYE2-OYE2ha #1-5

Even with the new-arriving primers, the Colony PCR fails.





*: 'C' means 'control', which is ADH6 from yeast genome 1

4. Transformation of ligation product.

TUESDAY, 2018-8-10

1. Colony PCR of pCRCT-OYE3/ADH6/ADH7

For each plate, 16 colonies was picked.

pCRCT-ADH6 and pCRCT-OYE3 shows correct bands and remaining PCR systems were sent for sequencing. pCRCT-OYE3 has been succesfully knockouted proving by the sequencing result on date 8.17.

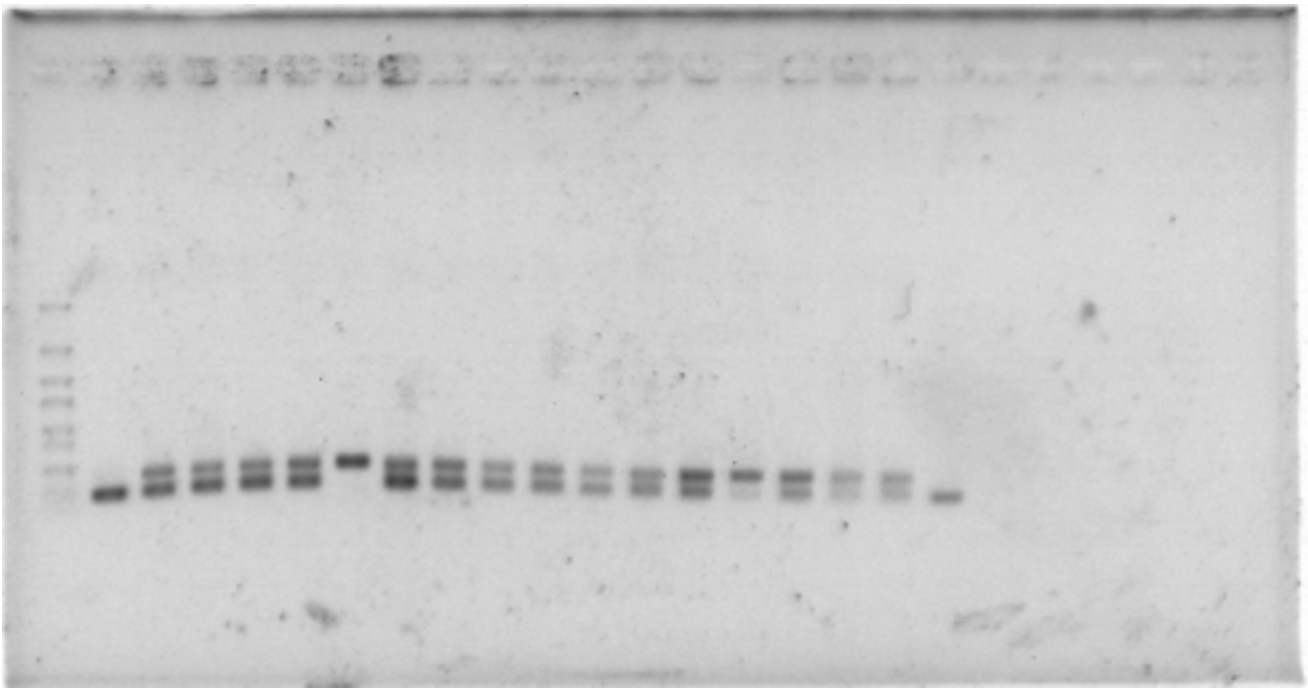
pCRCT-ADH7 shows 2 bands, with unknow reach one of the bands seem to be correct. Not sent for sequncing.

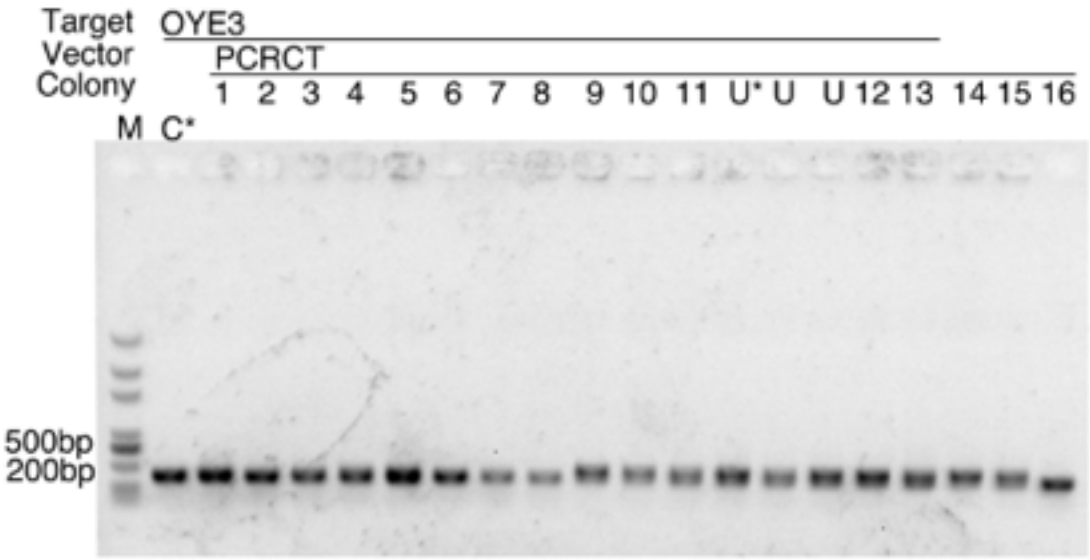
2. Colony PCR of traditional knockout

BY4741 was used as control, 2 pre-extracted genome and 2 glycerol stock were used.

6 colonies were picked for BY4741/ OYE2ha with Ura

7 colonies were picked for BY4741/ ATF1ha with Ura





*: 'C' means 'control', which is OYE3 from yeast genome 1

*: 'U' means 'Unknown', which is an unsure sample due to the researcher's slovenliness

20180810 trans5k BY4741-1-OYE2 BY4741-2-OYE2 genome1-OYE2 genome2-OYE2 OYE2-Ura-1~6 BY4741-1-ATF1 BY4741-2-ATF1 genome1-ATF1 genome2-ATF1 ATF1-Ura-1~6.Tif

All primer－dimers shown. Might due to operation error, thus the assay was repeated, still same result.

3. Colony PCR of BY4741/pCRCT-T1O2/T2O2

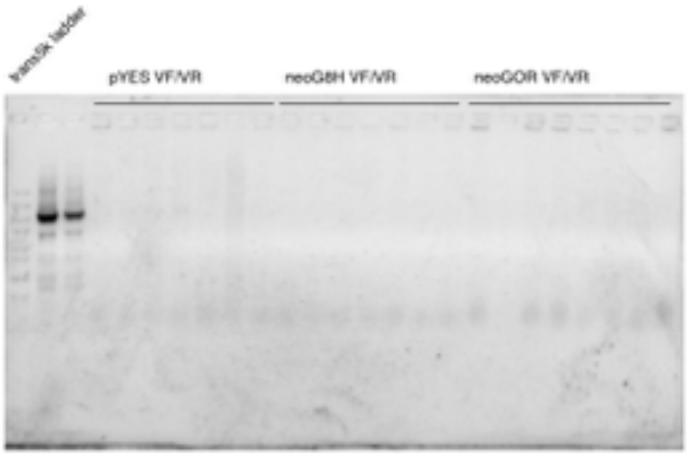
8 colonies were picked for each plasmid. ATF1 VF/VR and OYE2 VF/VR were used.

缺result

4. Colony PCR of pSB1C3-ISY-GOR and pYES-G8H

Fragment GOR G8H and pYES was tested using 3 pairs of primers

Only positive control of pYES were presented on the gel image.



SATURDAY, 2018-8-11

1. Use U-OYE2-F2/R2 to repeat Colony PCR of BY4741/ OYE2ha with Ura

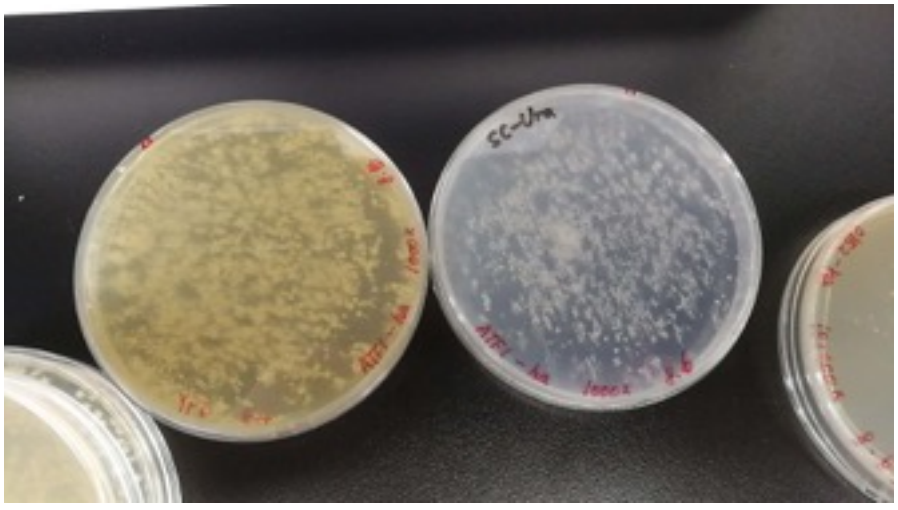
Positive result.

2. Transformation of Leu-ATF1 into Strain BY4741 and Strain BY4741△OYE2 with Ura

Main culture grew too slow, we guessed it might be affected as the pre-culture was placed as room tremperature for 1 day.

New preculture was made from -20°C glycerol stock.

3. photocopy BY4741△ATF1withURa/ATF1ha into both SC-Ura plate and YPD plate.



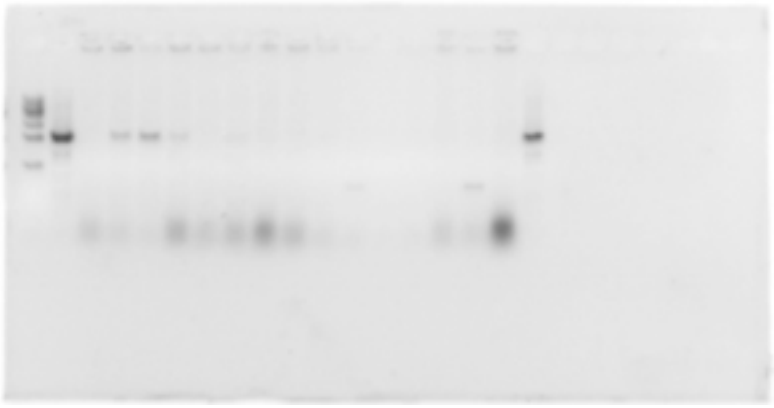
4. Plated the culture of BY4741/pCRCT-TnOn

4 tubes of TnOn was cultured in liquid SC-Ura for 2 days.

Diluted to 10^-3, 10^-4 and 10^-5, plated on SC-Ura dishes.

Glycerol stocks were made. #89 90 91 92

5. Redo Colony PCR of pSB1C3-ISY-GOR and pYES-G8H



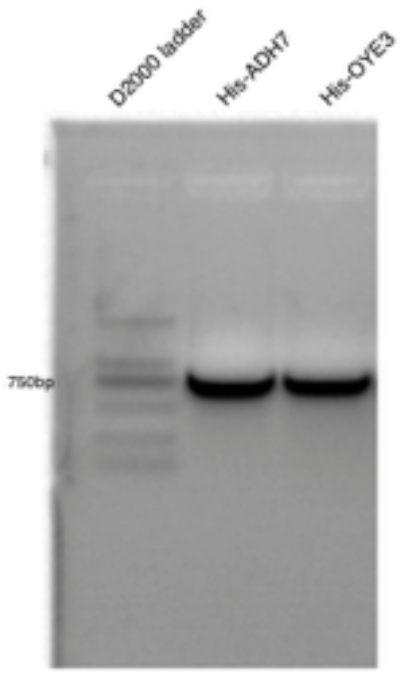
pSB1C3-ISY-GOR only has primer-dimers

pYES-G8H colony #23 has the same length as control

SATURDAY, 2018-8-13

1.Colony PCR of BY4741/pCRCT-ADH6 and BY4741/pCRCT-ADH7

2. Amplification of His-ADH7, His-OYE3



3, Genome extraction of BY4741

SUNDAY, 2018-8-14

1. **Plasmid PCR of pYES-G8H and pSB1C3-GOR-ISY**

   Same primers for colony PCR were used.

   For pYES-G8H plasmid #1-5 only primer dimers are shown

   pSB1C3-GOR-ISY has no bands.

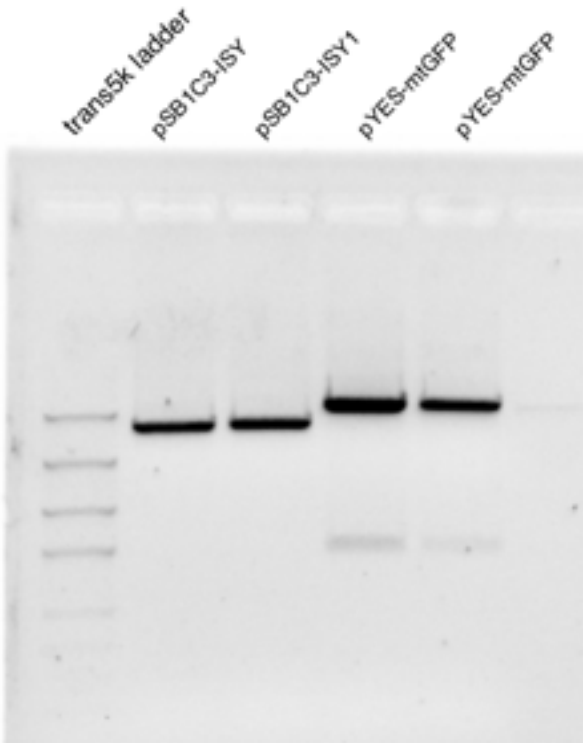
MONDAY, 2018-8-15

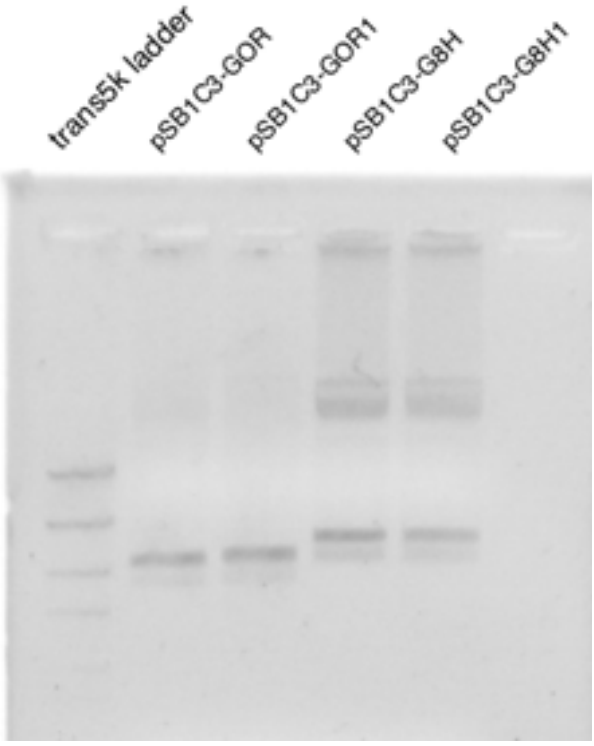
1. **Amplification of Leu-ATF1, gel extraction was performed.**
2. **Construction of pYES-ISY-G8H-GOR plan 2——RD and ligation**

   RD of pSC1C3-ISY using EcoRI and Xbal; RD of pSB1C3-GOR using EcoRI and SpeI

   RD of pSB1C3-G8H using SpeI and EcoRI; pYES-mtGFP using EcoRI and SpeI

   50μl RD system follows protocol. Ligation and transformation was performed follows protocol.





3. Preparation of 5-FOA plate

SDP+ Ura + 5-FOA	100ml
Yeast NB	1.7g
L-proline	1.0g
5-FOA	25mg
Uracil	10mg
glucose	20g
ddH2O	100ml

pH adjusted to 3.5~4

5-FOA 100x mother liquor : 25mg/μl desolved in DMSO

Filter-sterile

4. Preparation of SC-His plate

SC-His	300ml
Yeast NB	2.23g
-HIs	0.43g
20% glucose	(30ml)
ddH2O	270ml
Agar	6g

pH adjusted to 5,7

All Sterile.

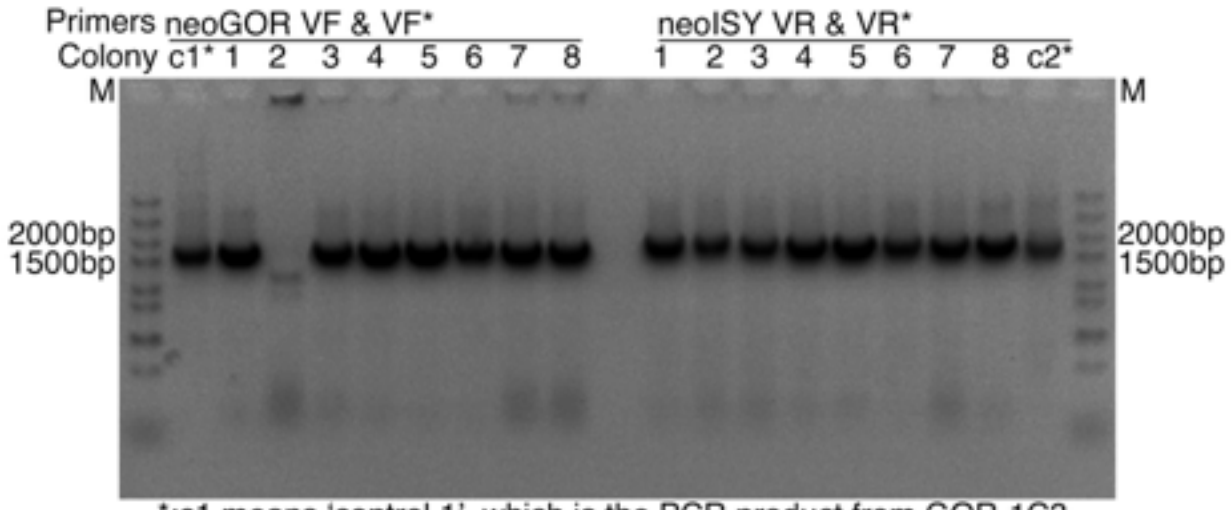
5.Transformation of Leu-ATF1 into BY4741△OYE2 with Ura competent cell made on the date 8.12

TUESDAY, 2018-8-16

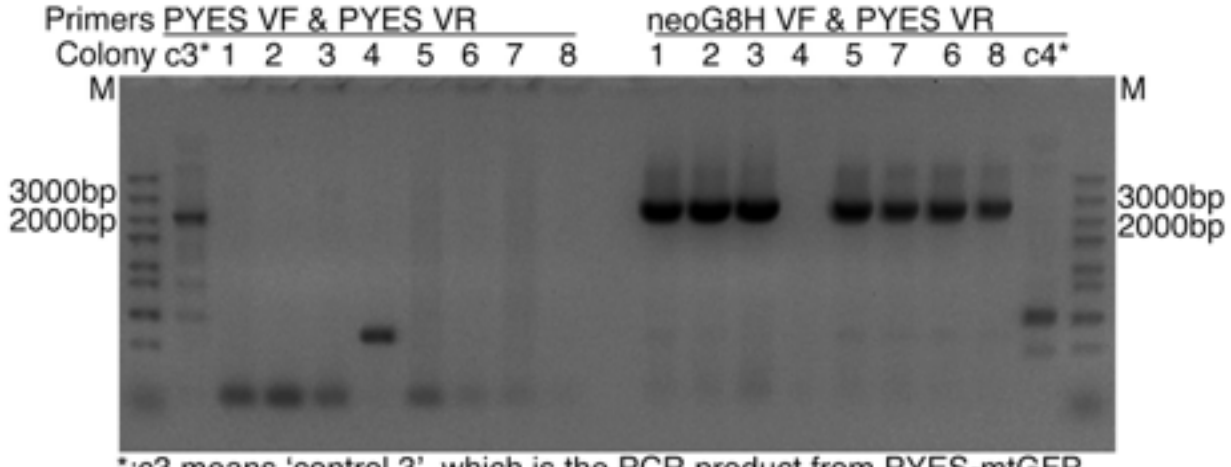
1. Colony PCR of pSB1C3-ISY-GOR and pYES-G8H

Fragment GOR G8H and pYES was tested using 4 pairs of primers.

8 colonies were picked from each plate.



*:c1 means 'control 1', which is the PCR product from GOR-1C3
*:c2 means 'control 2', which is the PCR product from ISY-1C3



*:c3 means 'control 3', which is the PCR product from PYES-mtGFP
*:c2 means 'control 2', which is the PCR product from PYES-mtGFP

pSB1C3-ISY-GOR colony #1 3 4 5 6 7 8 prepared for plasmid extraction.

pYES-G8H colony #1 3 5 were correct and prepared for plasmid extraction

2. Transformation of pSB4K5 from 2017kit, plate 4, 4D and pSB3C5 from 2018 kit, plate 4, 6H.

WEDNESDAY, 2018-8-17

1. **Colony PCR of pCRCT-OYE3 and pCRCT-ADH6**

   Colonies were picked from replicate plate, preparing more PCR system for sequencing.

THURSDAY, 2018-8-18

1. **Transformation of His-ADH7 and HIs-OYE3 into BY4741△OYE2 with Ura.**
2. **Innoculate pYES-G8H #1 3 5 and pSB1C3-ISY-GOR in 3ml LB broth with corresponding antibiotic.**
3. **Re-do the transformation of pSB4K5 from 2017kit, plate 4, 4D and pSB3C5 from 2018 kit, plate 4, 6H.**

FRIDAY, 2018-8-19

1. Plasmid miniprep of pYES-G8H #1 3 5 and pSB1C3-ISY-GOR. Glyderol stocks were made.

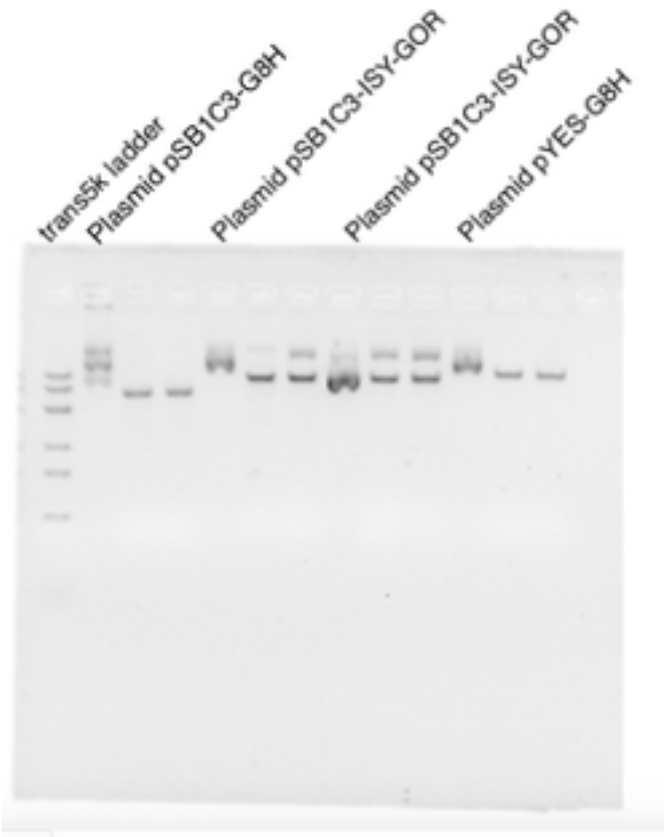
SATURDAY, 2018-8-20

1. **Making SC-His plates.**
2. **Praparation of 20% glucose. Sterilized.**
3. **Streaked Strain BY4741 on SC-Leu and SC-His**
4. **Plate BY4741/pCRCT-T2O2 on 5-FOA plates**

SUNDAY, 2018-8-21

1. Verifying the construction of pYES-G8H and pSB1C3-ISY-GOR

Plasmid were tested with 2 batch of Restriction enzymes.

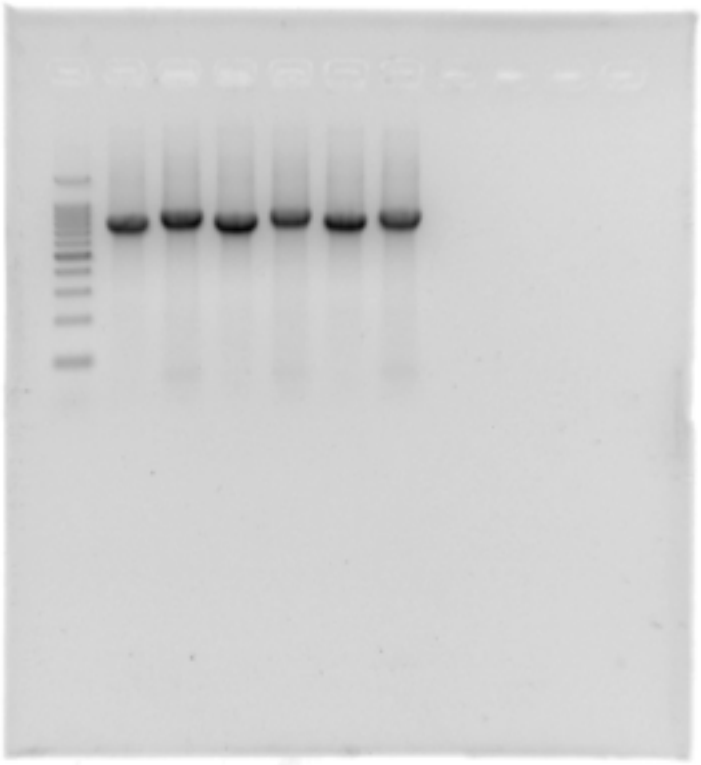


20180821 RD pls-1C3-G8H G8Hsp new G8Hsp old pls-ISY-GOR ISY-GORxp new ISY-GORxp old pls-ISY-GOR ISY-GORxs new ISY-GORxs old pls-PYES-G8H PYES-G8Hx new PYES-G8Hx old.Tif

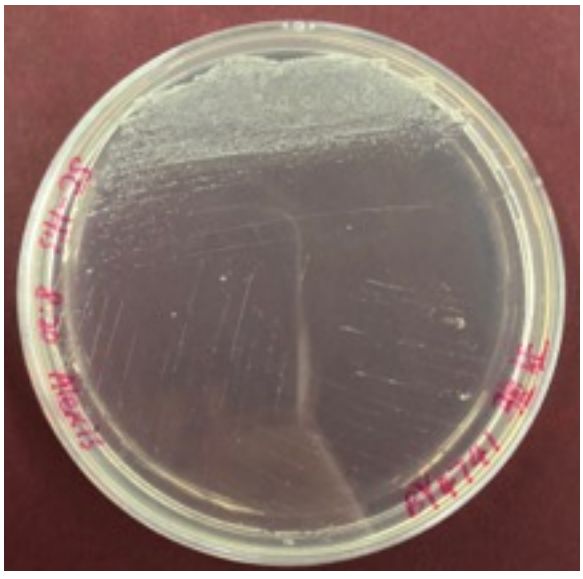
Old SpeI, XbaI and PtsI performed show equal efficiency as the new arriving ones.

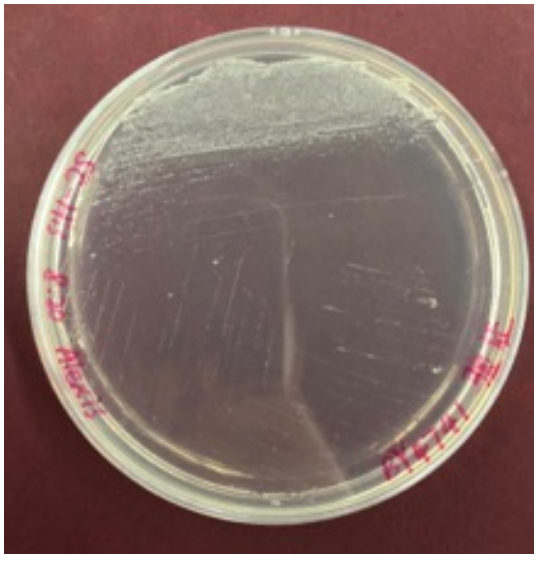
pYES-G8H show correct bands.However, the RD of pSB1C3-ISY-GOR shows unexpected bands. Thus we performed RD on the other 6 pre-extracted plasmid.

2.Redo verification of pSB1C3-ISY-GOR



3. Streaked Strain BY4741 on SC-Leu and SC-His





Having BY4741 grew on SC-His plate, we stopped using His in gene deletion by homogenous recombination. Only Ura and Leu can be used.

MONDAY, 2018-8-22

1. **Amplification of pYX223-Leu2 as verification**

WEDNESDAY, 2018-8-24

1. **Amplification and PCR product purification of Leu2-ATF1, 1C3-G8H and ISY-GOR**
2. **Transformation of Leu2-ATF1 into BY4741△OYE2 with Ura.**

   30min recovery in YPD broth.

MONDAY, 2018-8-27

1. **Transformation of proG8H-OYE2ha into BY4741△OYE2 with Ura for gene knock-in**
2. **Transformation of Leu2-ATF1 into BY4741△OYE2 with Ura.**

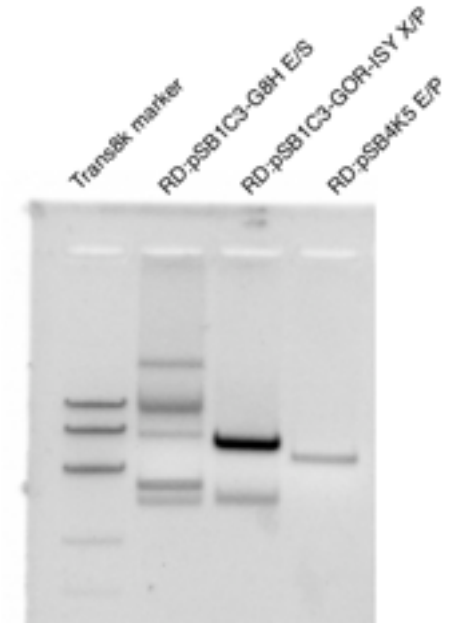
   Having learnt that yeast would not naturally carry out homogenous recombination unless insertion is longer than 5kb, we decided to integrate knockin and 5-FOA screening.
3. **Streaked BY4741△OYE2 with Ura on YPD plate**
4. **Overnight culture of pSB4K5, pSB1C3-GOR-ISY and pSB1C3-G8H in 5ml LB**

TUESDAY, 2018-8-28

1. **Plamid miniprep. of pSB4K5, pSB1C3-GOR-ISY and pSB1C3-G8H**

2. Construction of pSB4K5-G8H-GOR-ISY

RD：pSB4K5 using EcoRI and PstI; pSB1C3-G8H using EcoRI and SpeI; pSB1C3-GOR-ISY using XbaI and PstI.



Gel extraction, liagtion following protocols.

WEDNESDAY, 2018-8-29

1. Transformation of pSB4K5-G8H-GOR-ISY into BY4741, BY4741△OYE2 with Ura.

Competent cells were diluted and plated on YPD.

2. BY4741△OYE2 colony PCR to verify the discard of pCRCT-OYE2

BY4741△OYE2 containing pCRCT-OYE2 was inoculated in YPD broth and cultivated in 30 °C shaking incubator for 48h. The cultivated culture was diluted to 10^-3, 10^-4 and 10^-5, then plated on 5-FOA plate.

3. Knock in “promoter-G8H-OYE2ha” ——Streaked yawn on 5-FOA plate.

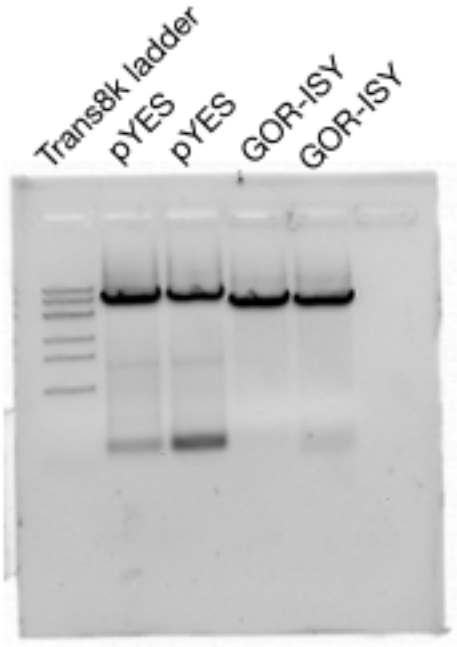
4. Pre-culture of BY4741△OYE2, BY4741△OYE2 with Ura and BY4741.

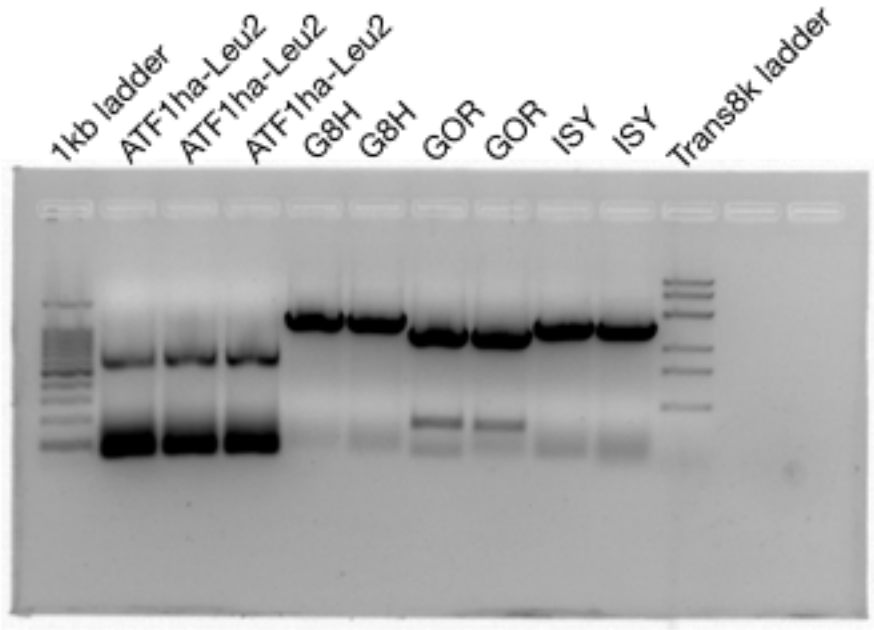
5. Preparation of 5-FOA plate.

THURSDAY, 2018-8-30

1. Amplification

Q5 2xmix was used.





Gel extractions were carried out.

2. GIbson assembly

Gibson assembly was performed to construct following plasmids:

pYES-G8H-GOR-ISY using 3 fragments: pYES, G8H, GOR-ISY.

pYES-G8H-GOR-ISY using 3 fragments: pYES, G8H, GOR and ISY

3. Transformation

pYES-G8H-GOR-ISY using 3 fragments: pYES, G8H, GOR-ISY. into Strain BY4741 and ` BY4741△OYE2

pYES-G8H-GOR-ISY using 3 fragments: pYES, G8H, GOR and ISY into Strain BY4741 and ` BY4741△OYE2

ATF1-Leu2 into BY4741△OYE2 with Ura.

4, Colony PCR of pSB1C3-ADH and pSB4K5-G8H-GOR-ISY

pSB1C3-ADH has colony#1 2 3 showing correct bands

pSB4K5-G8H-GOR-ISY has colony #8 showing bands of ISY only,

FRIDAY, 2018-8-31

1. Discarding pCRCT-OYE3 from BY4741△OYE3

The cultivated culture was diluted to 10^-3, 10^-4 and 10^-5, then plated on YPD and SC-Ura plate.

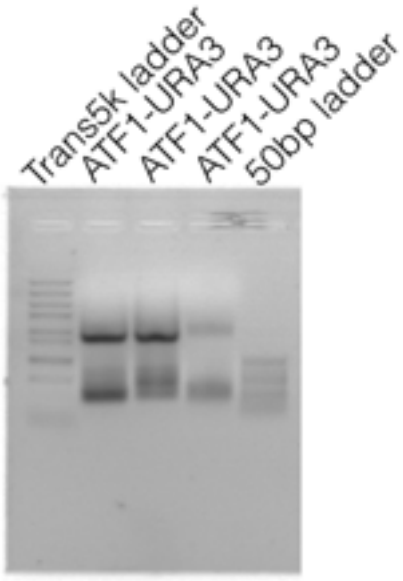
2. Discarding pCRCT-ADH6 from BY4741△ADH6

Correct colonies were picked from the replicate plate and streaked on 5-FOA plate

WEEK1，SEPTEMBER

1. Gene deletion

Amplification of ATF1-Ura3

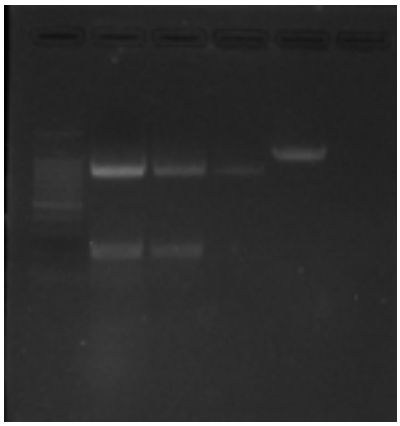


Transformation of ATF1-Ura3 and ATF1-Leu2(8.30) into BY4741△OYE2, Plated on SC-Ura/Leu plate.

Transformation of pCRCT－ADH7 / T1O2 / OYE3 into BY4741

` Transformation of Ura-OYE3/ATF1/ADH6/ADH7 into Strain BY4741△OYE2

2. Knock in of sequence used in optimization of metabolic pathway

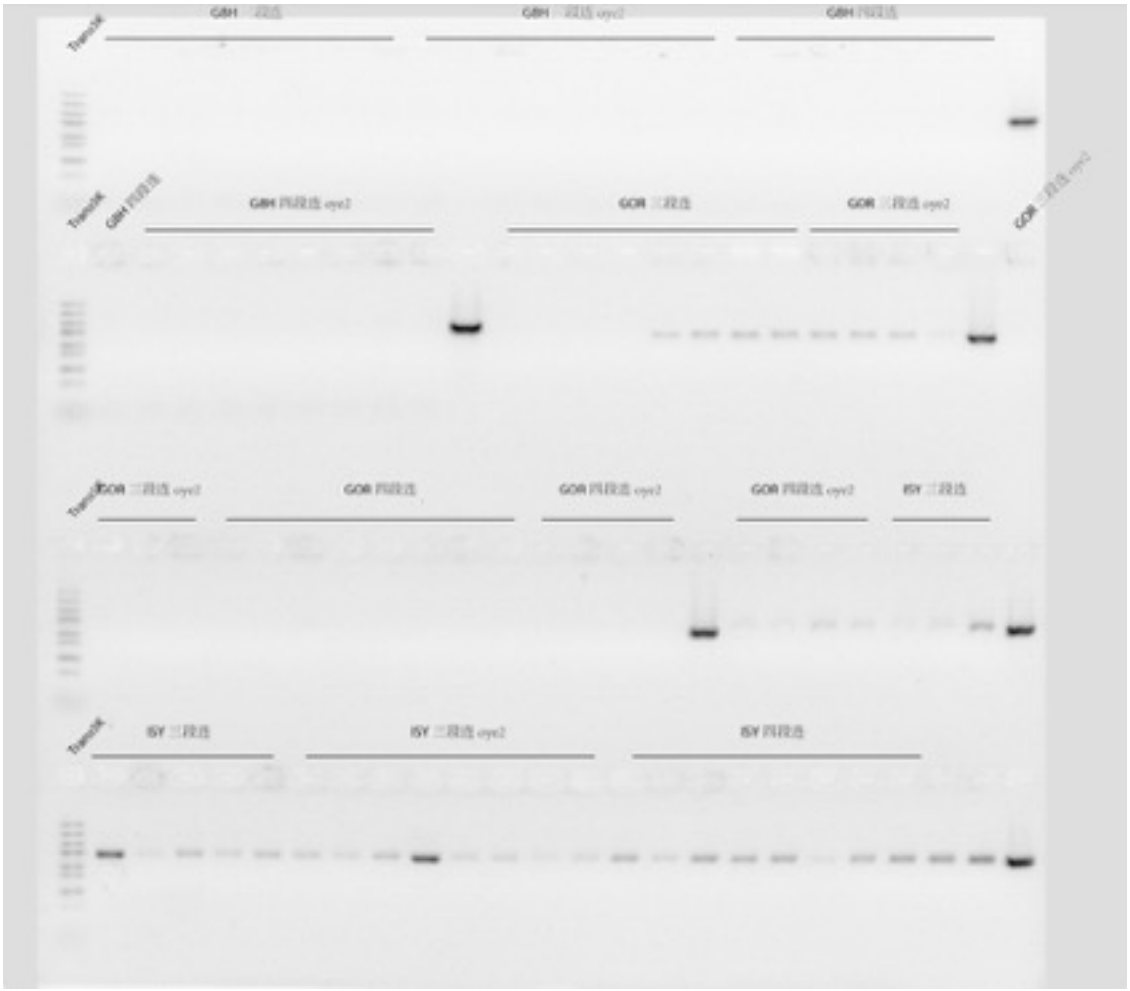


G8H-OYE2ha without promoter was failed to be amplified in first trial. Therefore we checked and re-designed the primers to achieve success. proG8H-OYE2ha were amplified in second trial, after adjusting to a lower annealing temperature.

Transformation of G8H-OYE2ha without promoter and proG8H-OYE2ha into BY4741△OYE2.

3. Construction of pYES-G8H-GOR-ISY

Plan 1: Initial plasmid design. Transform Gibson product into BY4741 directly.

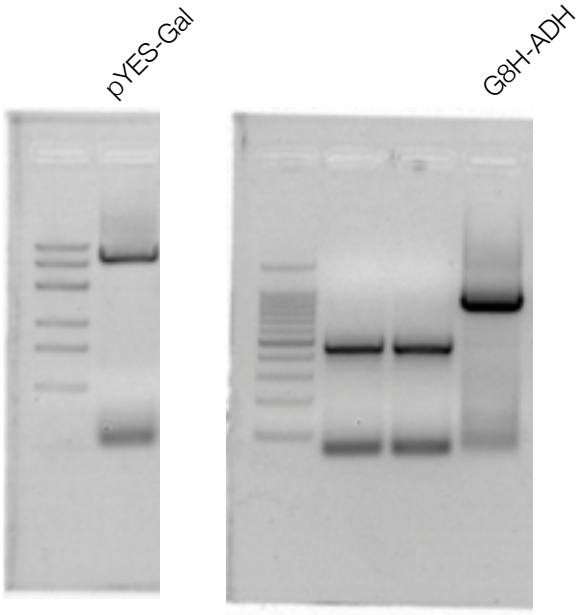


neoG8H VF/VR did not amplified any bands. it is possible that promoterTDH2 is replaced (due to the high transcriptase level of G8H), causing failure of PCR. New primer is thereby adopted to amplify coding regions

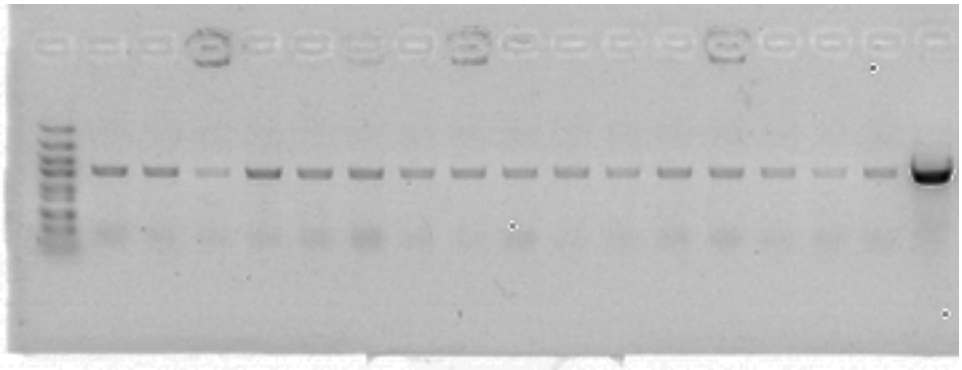
Plan 2: pYES-(Gal)-G8H-(ADH)-GOR-ISY

pSB1C3-G8H was assembled using RD(EcoRI&SpeI;EcoRI&Xbal) &ligation(T4 ligase).

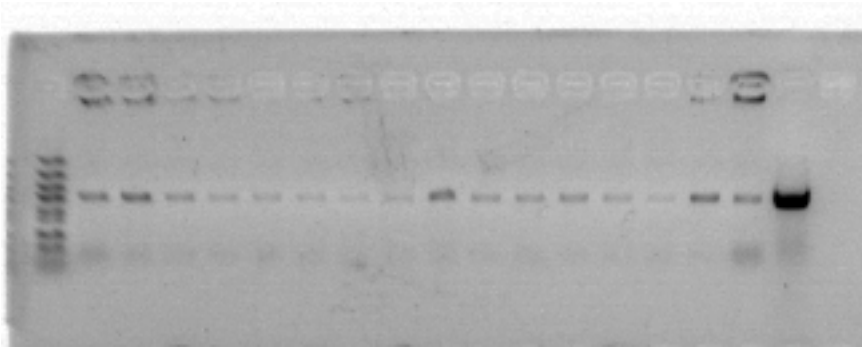
Gibson assembly of pYES-Gal, G8H-ADH and GOR-ISY. GOR-ISY were pre-amplified.



Colony PCR was carried out on 9.8, only G8H was checked as it has the least chance to be there. 16 colonies in total.



Plan 3: pYES-G8H and pYX142-GOR-ISY Co-transformation （abortion）



pYES-G8H colony PCR result shown as above. The plasmid is successfully assembled.

Plan 4: pYES-TDE2-G8H-GOR-ISY （abortion）

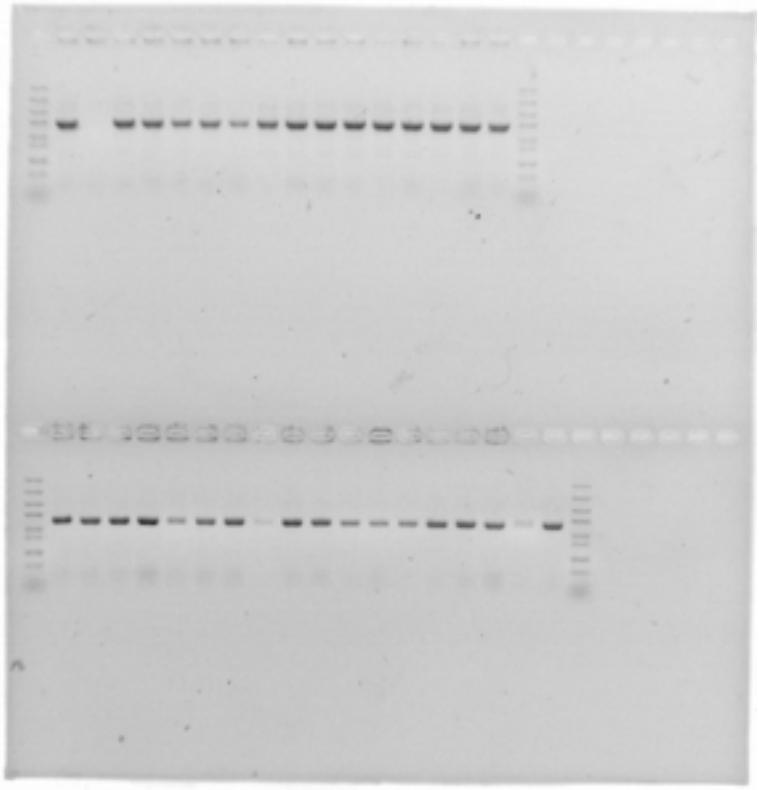
4. Testing Gas chromatography condition of Nepetalactol

8-hydroxygeraniol, 8-oxogeraniol and nepetalactol were prepared in the conc. of 1mg/L, 5mg/L, 10mg/L, 50mg/L and 100mg/L as standard references. No peak of standard references due to chromatographic column temperature was not high enough.

WEEK2，SEPTEMBER

1. Redo the colony PCR of BY4741△OYE2 containing pYES-G8H-GOR-ISY

We amplified the 2 remaining fragments of pYES-G8H-GOR-ISY. Templates are colonies from the replicate plate. The upper row uses neoISYVF/VR and the lower row uses neoGORVF/VR.



2. BY4741△OYE2 containing plasmid of interest was diluted and plated on YPD plate

pCRCT-ADH6	pCRCT-OYE3
pCRCT-ADH7	pCRCT-T1O2

4. Testing Gas chromatography condition of Nepetalactol

The peaks of Nepetalactol were detected after adopting the new program, though it did not present the change in peak area when the conc. of standard references vary.

Hydroxygeraniol and oxogeraniol can be detected, but the difference in between was not shown by Gas Chromatography.

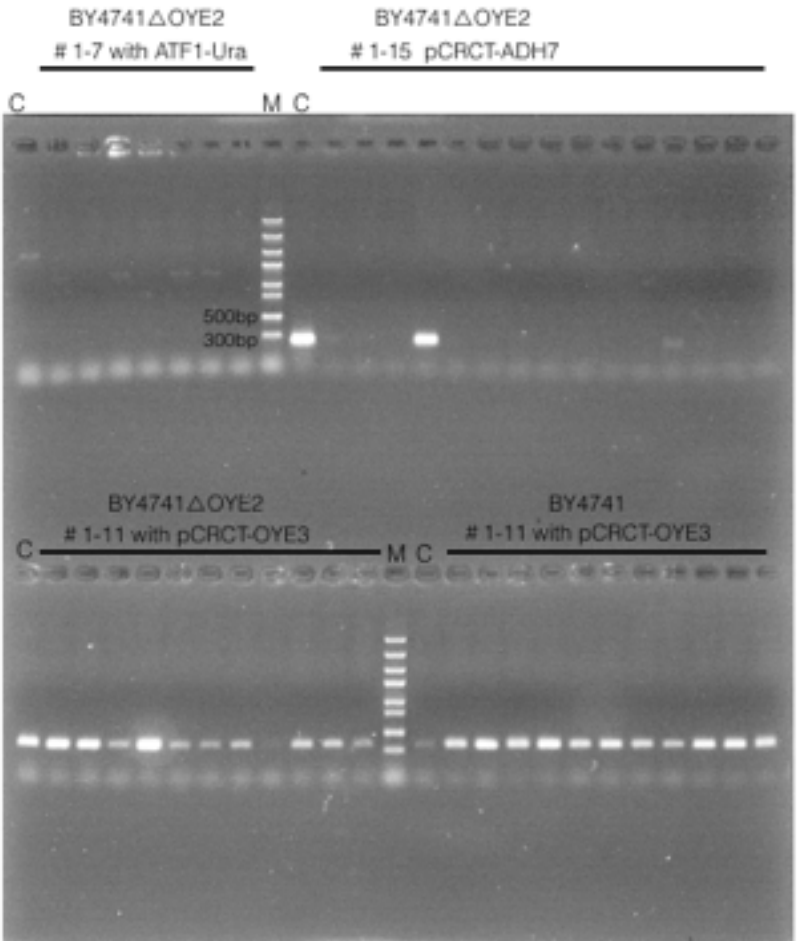
5. Gene Deletion

- BY4741△OYE2 containing plasmid of interest was diluted and plated on YPD plate

pCRCT-ADH6 pCRCT-OYE3

pCRCT-ADH7 pCRCT-T1O2

-Colony PCR



BY4741△OYE2 with pCRCT-OYE3 were sequenced and colonies #1-6 achieved the success of deleting OYE3.

Strain BY4741△OYE2△OYE3 is obtained.

WEEK3，SEPTEMBER

1. Flask–shake fermentation of nepetalactol

9.17 Inoculation of BY4741△OYE2/pYES-G8H-GOR, BY4741△OYE2/pYES-(Gal)-G8H-(ADH)-GOR, BY4741/pYES-G8H-GOR, BY4741/pYES-(Gal)-G8H-(ADH)-GOR into 3ml YPD broth as pre-culture. For each strain, 2 free colonies were picked.

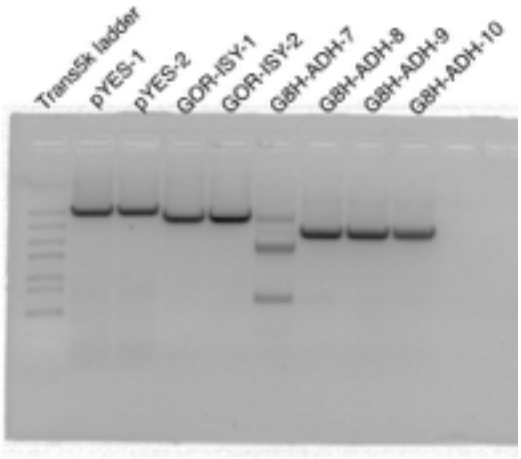
9.18 Dilution of pre-culture: Inculcate 100μL of each cultivated culture into 30ml of SC-Ura. Grew at 30°C , 250rpm for 24h.

9.19 Add 15μL of 200mM geraniol-ethanol solution at the beginning. Grow at 28˚C, 250r.p.m for 24 hours.

9.20` Add 0.75μL of 200mM geraniol-ethanol solution each 1 hour for 20 hours

9.21 Sample preparation for Gas chromatography

#	Strain and plasmid	medium	Retention time	peak area
Geraniol standard ref. 1	/	/	4.259	3606
Geraniol standard ref. 2	/	/	4.258	10442
Geraniol standard ref. 3	/	/	4.257	21851
Geraniol standard ref. 4	/	/	4.259	97683
Geraniol standard ref. 5	/	/	4.258	113568
Nepetalactone standard ref. 1	/	/	4.658	2384
Nepetalactone standard ref. 2	/	/	4.657	5216
Nepetalactone standard ref. 3	/	/	4.657	6206
Nepetalactone standard ref. 4	/	/	4.657	21843
Nepetalactone standard ref. 5	/	/	4.660	42307
Negative control			4.257	/
Sample1	4741 pyes	SC－ura	4.272	67651
Sample2	4741 pyes	SC－ura	4.271	89065
Sample3	oye2 pyes	SC－ura	4.270	92620
Sample4	oye2 pyes	SC－ura	4.269	106036
Sample5	oye2 gal pyes	SC－ura	4.268	107176
Sample6	oye2 gal pyes	SC－ura	4.267	106812
Sample7	4741 pyes	YPD	4.267	61026
Sample8	4741 pyes	YPD	4.267	31808
Sample9	oye2 pyes	YPD	4.267	55407
Sample10	oye2 pyes	YPD	4.264	49065
Sample11	oye2 gal pyes	YPD	4.265	95830
Sample12	oye2 gal pyes	YPD	4.262	106922



2. Gene deletion

-Plasmid discard of Strain BY4741△OYE2△OYE3

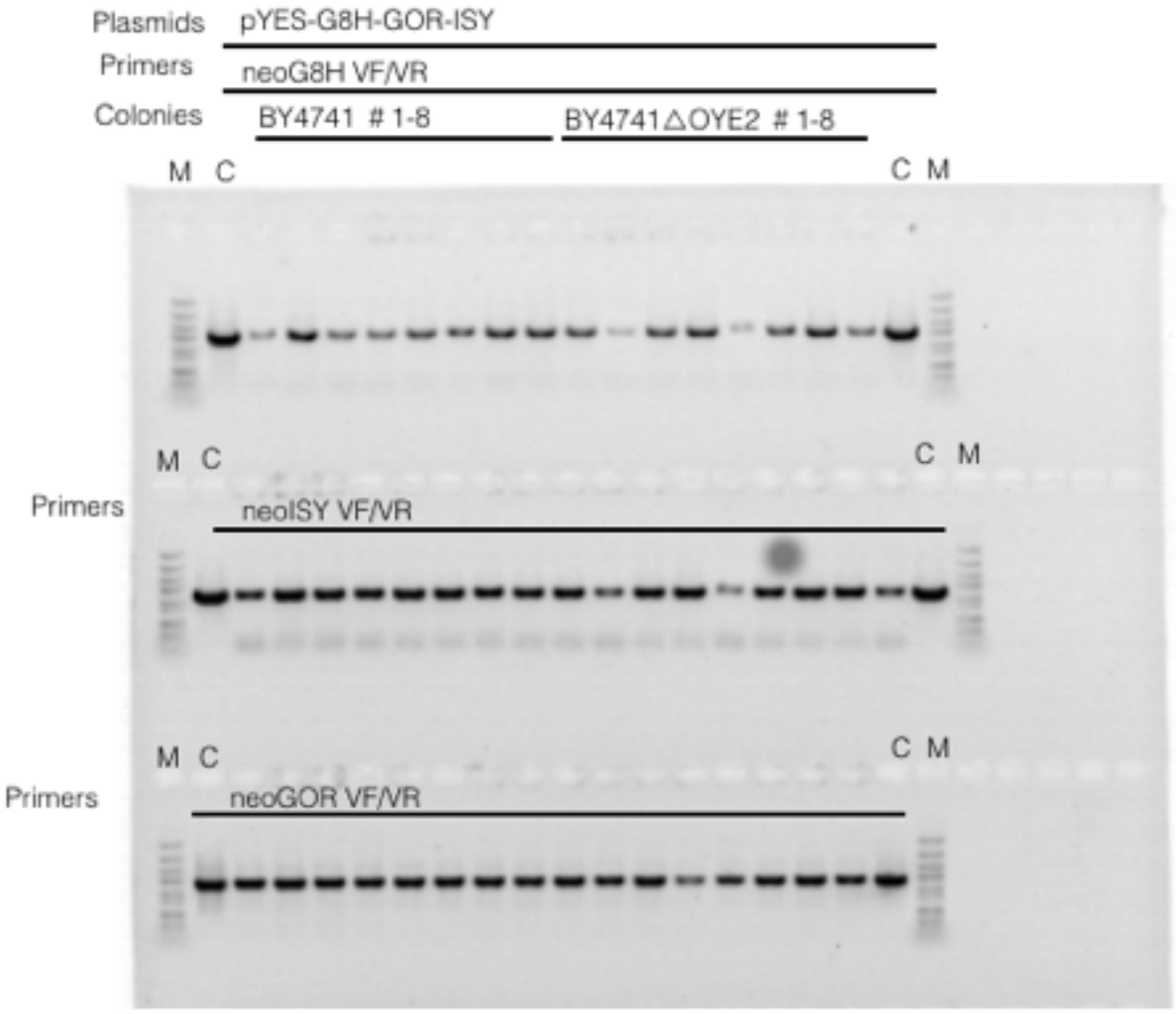
3. Construction of metabolic pathway

9.22 Amplification of fragments followed by Golden gate assembly and transformed into BY4741 and BY4741△OYE2

WEEK4，SEPTEMBER

1. Construction of metabolic pathway

Colony PCR of *BY4741△OYE2*/pYES-G8H-(ADH)-GOR-ISY and *BY4741△OYE2*/pYES-G8H-GOR-ISY.



*C refers to positive control
*M refers to Trans8kladder

2. Flask–shake fermentation of nepetalactol

9.25 Inoculation of BY4741/PYES-G8H-GOR-ISY into 3ml SC-Ura as pre-culture

Plasmid extraction performed and Transformed into BY4741△OYE2.

9.26 Dilution of pre-culture: Inculcate 100μL of each cultivated culture into 30ml of SC-Ura. Grew at 30°C , 250rpm for 24h.

9.27 Add 200μL of 200mM geraniol-ethanol solution at the beginning. Grow at 28°C, 250r.p.m for 24 hours.

9.28 24h sample of BY4741/PYES-G8H-GOR-ISY was collected. Wrapped with parafilm and store at -4°C freezer.

Colony PCR of BY4741△OYE2/PYES-G8H-GOR-ISY——all correct

Pre-culture was prepared.

9.29-30 GC was carried out.

Result: No nepetalactol was detected by GC. Colony PCR will be carry out to testify the validity of Plasmid. Minor geraniol consumption.

	GC	9.29		
#	Strain	Plasmid	Retention time	peak area
Geraniol standard ref. 1	/	/	4.249	4615
Geraniol standard ref. 2	/	/	4.246	13619
Geraniol standard ref. 3	/	/	4.244	27835
Geraniol standard ref. 4	/	/	4.242	72815
Geraniol standard ref. 5	/	/	4.241	104143
Nepetalactone standard ref. 1	/	/	4.646	2851
Nepetalactone standard ref. 2	/	/	4.646	3854
Nepetalactone standard ref. 3	/	/	4.647	21780
Nepetalactone standard ref. 4	/	/	4.650	15241
Nepetalactone standard ref. 5	/	/	4.653	42307
Negative control 1	BY4741		4.245	755184
Negative control 2	BY4741△OYE2		4.244	704655
Negative control 3	BY4741△OYE2△OYE3		4.244	612825
Sample 1	BY4741	pYES-G8H-GOR-ISY-1	4.243	652559
Sample 2	BY4741	pYES-G8H-GOR-ISY-2	4.243	383723
Sample 3	BY4741	pYES-G8H-GOR-IS-3	4.244	607897
Sample 4	BY4741△OYE2	pYES-G8H-GOR-ISY-1	4.244	725771
Sample 5	BY4741△OYE2	pYES-G8H-GOR-ISY-2	4.244	137717
Sample 6	BY4741△OYE2	pYES-G8H-GOR-IS-3	4.243	655485

3. Gene Deletion

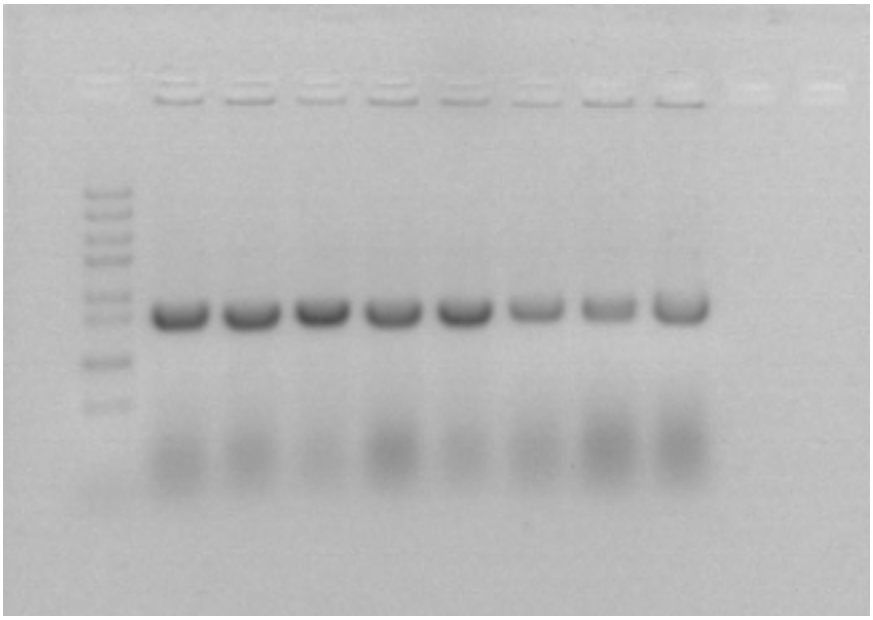
– Construction of pCRCT-ATF1

9.25 Amplification and Gel extraction of ATF1-gg and pCRCTbackbone-gg.

Golden gate assembly was performed

9.26 transformation into DH5α.

9.28 Colony PCR



9.29 Plasmid extraction

- pCRCT-ADH6/ADH7

9.25 Transformation of pCRCT-ADH6 and pCRCT-ADH7 into BY4741△OYE2△OYE3

WEEK1&2, OCTOBER

1. Co-culture

Streaked BY4741△OYE2△OYE3/pYES-G8H-GOR-ISY and DH5α on YPD plate and LB plate(K+C)

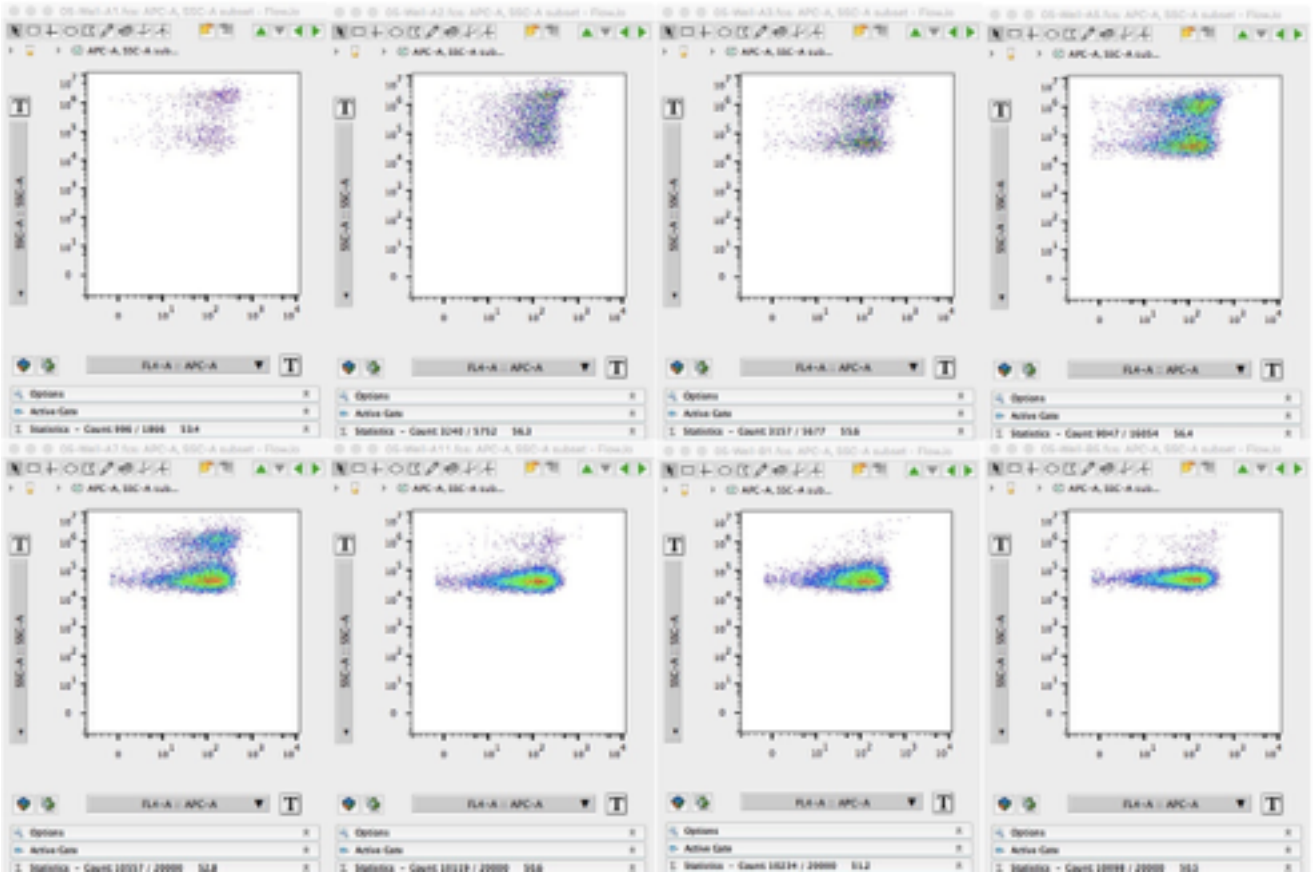
Inoculation 4 free colonies into co-culture broth for 24h

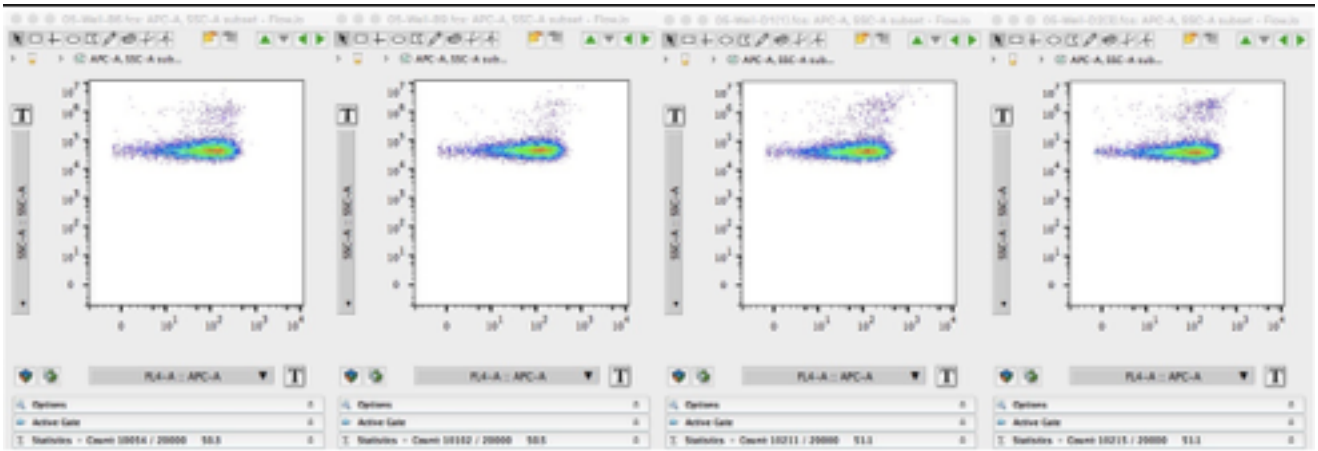
Dilute into 50ml Co-culture broth and shake at 30°C

Sampling was performed every 2h: Pippet 20μl cultivated culture into 180μl 1x PBS

Dilute to 10^-4 and pippet 50μl to plate on LB plate(with antibiotic and without antibiotics)

Add 1.5μl Kanamycin into remaining 10^-4 cultivated culture, labeled and stored at -4°C freezer.





2. Flask-shake fermentation of Nepetalactol

Colony PCR was performed on Strains involved in last trial of fermentation, only ISY was succesfully amplified.

Inoculation of BY4741△OYE2 in 3ml broth as pre-culture.

Plasmid extraction of pYES-(Gal)-G8H-GOR-ISY from BY4741

Amplification was performed on the plasmid to check it’s validity. Positive result

Transformation of pYES-(Gal)-G8H-GOR-ISY into DH5a and BY4741△OYE2

The latter transformation has failed, but the former one has colonies that contains plasmid of interest.

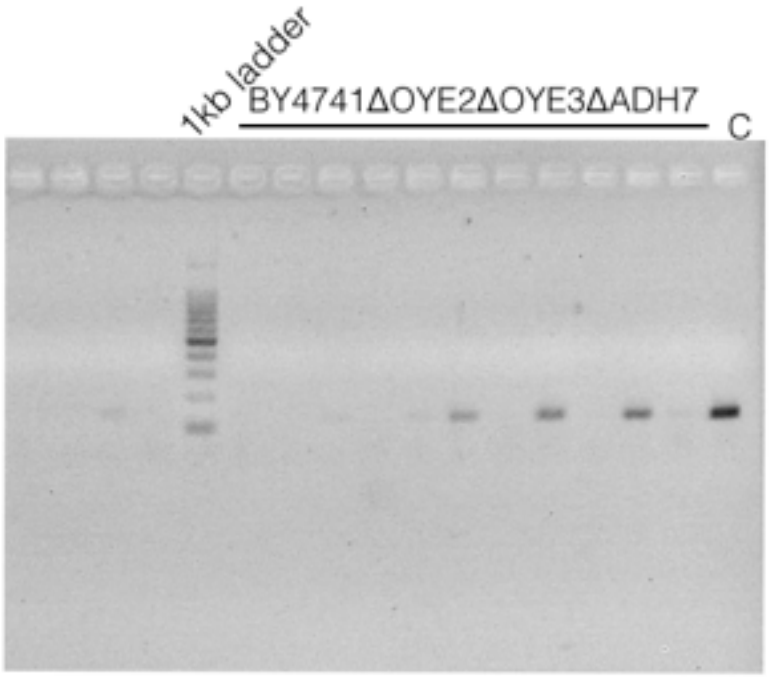
Thus, plasmid extraction were performed on DH5a and followed by transformation into BY4741△OYE2.

After 48h of incubation, colonies grew on the plate.

3. Gene deletion

10.1-10.6 Cell passage was carried out

10.9 Colony PCR of BY4741△OYE2△OYE3 transformed pCRCT-ADH7



lane#19 21 22 were sent for sequencing and back with positive result.

Colony will be performed with plasmid discard.