Notebook of Deleting Genes and Assembling Plasmids

Week one:2018-07-09 to 2018-07-15

Deletion of BARI

construct the gRNA plasmid,instruct by the following protocol:

1)Adhesion to gain gRNA

Volume of 20μL

oiHB063(100mM)	9μL
oiHB064(100mM)	9μL
10×Annealing buffer	2μL

The PCR procedure:

95℃ 5min

90℃ 15s ┐

├ Minus 5 degrees for each cycle，14cycles.

90℃ 15s ┘

20℃ ∞

Notice:oiHB063 and oiHB064 are two complementary single stranded primers which are 60bp length. And Homologous sequences of 20 bp were found in the upstream and downstream of plasmid NotI site.The middle 20bp is the sequence of the protospacer adjacent motif in the target area to be cut and adjacent to the base sequence. The sequence of the first 20bp is the protospacer.

2)We inoculate the bacteria in the culture medium to let it grow. When they After reproducing to the right concentration, we extract the plasmid pxzx538、pxzx400 out of the cell.

3)To get the linearized plasmid,we use the NotI enzyme to cut the plasmid. The protocol is as follow.

plasmid	70μL
10×cutsmart	8.1μL
NotI	2.5μL

37℃ water bath 90min.

4)After the water bath, we get the linearized plasmid. To test whether the linearized plasmid is right, we have to ionophoresis the sample in the 1% agarose gel. If the length is right, cut that part of gal and purify it to obtain the right linearized plasmid.

5)Construct the gRNA and the linearized plasmid - Gibson Assembly

The protocol is as follow:

5×SE cloning buffer	2μL
gRNA	0.5μL
pxzx400	6.5μL
SE Recombinase	1μL
total	10μL

Place the mixture in the 37°C incubator for 1 hour.

Notice:The products that Gibson assembled needed to be used at once, can not be long reset.

Week two:2018-07-16 to 2018-07-22

1)After the Gibson assembly, we got the integrated plasmid. Then we transform it to the colibacillus to amplify the plasmid.
2)When the colibacillus grow into colonies, select four colonies smear to the Sc-ura solid medium.
3) After the colibacillus grows to a certain density, we mix the cell with the liquid medium, waiting for it to grow in to a certain density, then we can extract the plasmid out of the colibacillus and send it to sequencing.
4)According to the sequencing result, we choose the No.2 strain of pxzx400, then mix the colibacillus in the 50% glycerinum and store it in the -80°C refrigerator.
5)Using three PCR reaction systems to obtain the fragments with homology arms. The system is as below:

ddH2O	8.5μL
2×phanta Max Buffer	12.5μL
dNTP Mix(10mM)	0.5μL
Upstream primer	1μL
Downstream primer	1μL
phanta Max Super-Fidelity DNA polymerase	0.5μL
Template DNA	1μL

6)After pcr, we fetch 10μL sample mixed with 2μL loading buffer, agarose gel electrophoresis to examine whether the length is right.
7)We fetch 50μL sample to do the overlap PCR. Take each of the three mixed systems obtained in step (1) as the template, a total of 3μL, or the mixed system obtained in step (3) as the template, and take the sample of 1μL, p 50μL large sample.
The system of the overlap PCR is as below:

ddH2O	16μL
2×phanta Max Buffer	25μL
dNTP Mix(10mM)	1μL
Upstream primer	2μL
Downstream primer	2μL
phanta Max Super-Fidelity DNA polymerase	1μL
Template DNA	3μL

After overlap PCR, we obtain the produce mixture. So we have to purify it by agarose gel electrophoresis.

Week three:2018-07-23 to 2018-07-29

1)Transformation of yeast: Transform the plasmid with gRNA into the strain whose

Gal4 and Gal80 genes has been deleted, whose plasmid with ura label das been lost, but still has the plasmid with Cas9 in.

Notice: This time, we set the control group as transforming the plasmid labeled with gRNA into BY4741. And another way is transforming the plasmids labeled with gRNA and Cas9 into BY4741.

2) When the yeast grow up on the culture medium, select the single colonies and culture by the streak method on the solid media. Culture in 30°C incubator overnight.

3)PCR Validation: Colony PCR

Boil the cell in 20mM NaOH (50μL) with the following program.

99℃ 5min
4℃ 1min
} 3cycles

Then use the boiled cell as the template, prepare the solution system as followed:

template	1μL
Upstream primer	0.3μL
Downstream primer	0.3μL
10×TransFast Taq Buffer	1.5μL
2.5mM dNTPs	1.2μL
TransFast Taq DNA Polymerase	0.3μL
ddH2O	10.4μL

Do the PCR validation with the following program:

94℃ 3min
94℃ 5s
52℃ 15s
72℃ 30s
} 30 cycles
72℃ 7min

Then select the right single colony and culture by the streak method on a new solid media for future validation.

4)When the cell grown up, select the single colony and culture by streak method on the solid media. When that cell grows up, validate them by colony PCR. Using three pairs of primers to validate whether the Gal4、Gal80 and BARI gene have been deleted. After obtaining the right strain, lost the plasmid and store it with glycerinum method at -80°C.

Week four:2018-07-30 to 2018-08-05

Deletion of Gal4and Gal80

Assemble two gRNA fragments with pxzx400 plasmid,instruct by the following protocol:

1)Adhesion to gain gRNA

Volume of 20μL

oiHB063(100mM)	9μL
oiHB064(100mM)	9μL
10×Annealing buffer	2μL

The PCR procedure:

95℃ 5min

90℃ 15s ┐

90℃ 15s ┘ Minus 5 degrees for each cycle，14cycles.

20℃ ∞

2)We inoculate the bacteria in the culture medium to let it grow. When they After reproducing to the right concentration, we extract the plasmid pxzx400 out of the cell.

3)To get the linearized plasmid,we use the NotI enzyme to cut the plasmid. The protocol is as followed.

plasmid	70μL
10×cutsmart	8.1μL
NotI	2.5μL

37℃ water bath 90min.

4)After the water bath, we get the linearized plasmid. To test whether the linearized plasmid is right, we have to ionophoresis the sample in the 1% agarose gel. If the length is right, cut that part of gal and purify it to obtain the right linearized plasmid.

5)Assemble the gRNA and the linearized plasmid - Gibson Assembly

The protocol is as follow:

5×SE cloning buffer	2μL
gRNA	0.5μL
pxzx400	6.5μL
SE Recombinase	1μL
total	10μL

Place the mixture in the 37°C incubator for 1 hour.

Notice:The products that Gibson assembled needed to be used at once, can not be long reset.

6)And we assemble another gRNA to the same plasmid which is cut by HindⅢ enzyme, using the same program.

plasmid	70μL
10×cutsmart	8.1μL
HindⅢ	2.5μL

Week five:2018-08-06 to 2018-08-12

1)After the Gibson assembly, we got the integrated plasmid. Then we transform it to the colibacillus to amplify the plasmid.

2)When the colibacillus grow into colonies, select four colonies and culture by streak method on the Sc-ura solid medium.

3) After the colibacillus grows to a certain density, we mix the cell with the Sc-Leu liquid medium, waiting for it to grow in to a certain density, then we can extract the

plasmid out of the colibacillus and send it to sequencing.
4)According to the sequencing result, we choose the No.2 strain of pxzx400, then mix the colibacillus in the 50% glycerinum and store it in the -80°C refrigerator.
5)Using three PCR reaction systems to obtain the fragments with homology arms.
The system is as below:

ddH2O	8.5μL
2×phanta Max Buffer	12.5μL
dNTP Mix(10mM)	0.5μL
Upstream primer	1μL
Downstream primer	1μL
phanta Max Super-Fidelity DNA polymerase	0.5μL
Template DNA	1μL

6)After pcr, we fetch 10μL sample mixed with 2μL loading buffer, agarose gel electrophoresis to examine whether the length is right.
7)We fetch 50μL sample to do the overlap PCR. Take each of the three mixed systems obtained in step (1) as the template, a total of 3μL, or the mixed system obtained in step (3) as the template, and take the sample of 1μL, pcr for 50μL large sample.
The system of the overlap PCR is as below:

ddH2O	16μL
2×phanta Max Buffer	25μL
dNTP Mix(10mM)	1μL
Upstream primer	2μL
Downstream primer	2μL
phanta Max Super-Fidelity DNA polymerase	1μL
Template DNA	3μL

After overlap PCR, we obtain the produce mixture. So we have to purify it by agarose gel electrophoresis.

Week six:2018-08-13 to 2018-08-19

The selection of the promoters
1)Transformation of yeast: Transform the plasmid with gRNA into the strain whose Gal4 and Gal80 genes has been deleted, whose plasmid with ura label das been lost, but still has the plasmid with Cas9 in.
Notice: This time, we set the control group as transforming the plasmid labeled with gRNA into BY4741. And another way is transforming the plasmids labeled with gRNA and Cas9 into BY4741.
2) When the yeast grow up on the culture medium, select the single colonies and culture by the streak method on the solid media. Culture in 30°C incubator overnight.
3) PCR Validation: Colony PCR

Boil the cell in 20mM NaOH (50μL) with the following program.

99℃ 5min
4℃ 1min
} 3cycles

Then use the boiled cell as the template, prepare the solution system as followed:

template	1μL
Upstream primer	0.3μL
Downstream primer	0.3μL
10×TransFast Taq Buffer	1.5μL
2.5mM dNTPs	1.2μL
TransFast Taq DNA Polymerase	0.3μL
ddH2O	10.4μL

Do the PCR validation with the following program:

94℃ 3min
94℃ 5s
Tm-3℃ 15s
72℃ 30s
} 30 cycles
72℃ 7min

Then select the right single colony and culture by the streak method on a new solid media for future validation.

4)When the cell grown up, select the single colony and culture by streak method on the solid media. When that cell grows up, validate them by colony PCR. Using primers to validate whether the Gal4、Gal80 and Gal4,Gal80 gene have been deleted. After obtaining the right strain, lost the plasmid and store it with glycerinum method at -80°C.

Week seven:2018-08-20 to 2018-08-26

The selection of the report genes

1) We choose to measure three different promoter's intension: TDH3P,TEF1P and PGK1P. To characterize the promoter's intension, we design to attach fluorescent protein mCherry after the under tested promoter.

2) First, we have to obtaining three pairs of fragments with homology arms. Instruct by the following protocol:

Volume of 50μL

Phanta max buffer(2X)	25μL
primers	2μL each
template	1μL genome or plasmid
dNTP mix(10mM)	1μL
Phanta max super-fidelity DNA polymerase	1μL

The PCR program are as followed:

For PGK1T

95℃ 3min

95℃ 15s
53℃ 15s ├ 30 cycles
72℃ 30s

72℃ 5min

ForPGK1P

95℃ 3min

95℃ 15s
47℃ 15s ├ 30 cycles
72℃ 30s

72℃ 5min

For TEF1T

95℃ 3min

95℃ 15s
53℃ 15s ├ 30 cycles
72℃ 30s

72℃ 5min

For TEF1P

95℃ 3min

95℃ 15s
47℃ 15s ├ 30 cycles
72℃ 30s

72℃ 5min

After PCR, we obtain the purified DNA by extracting it from gel using DNA extraction kit.

3) We attach the promoter,fluorescent protein and the terminator together. We choose the Yeast homologous recombination method to do this. We transform the fragments into yeast.you can find the yeast transformation's protocol in our wiki.

4) When the transformed yeast grow up, we validate them by colony PCR,youcan find the protocol in our wiki.

5 ） after we validate the germ is right, we measure the fluorescence intensity by microplate reader to compare three different promoters.

Week eight:2018-08-27 to 2018-09-02

The selection of the report genes

Aims to find a suitable reporter gene,we choose three fluorescent protein:EYFP,mCherry and mOrange. We attach the fluorescent protein gene after TDH3P promoter. The protocol for this experiment is as below:

5) First, we have to obtaining promoter,reporter gene and terminator fragments with homology arms. Instruct by the following protocol:

Volume of 50μL

Phanta max buffer(2X)	25μL
primers	2μL each
template	1μL
dNTP mix(10mM)	1μL
Phanta max super-fidelity DNA polymerase	1μL

The PCR program is as followed:
95℃ 3min
95℃ 15s
Tm-3℃ 15s ┐ 30 cycles
72℃ 30s ┘
72℃ 5min

6) We attach the promoter,fluorescent protein and the terminator together and assemble the cassette onto the pRS413 plasmid. We choose the Yeast homologous recombination method to do this. We transform the fragments and pRS413 plasmid into yeast.You can find the yeast transformation's protocol in our wiki.

7) When the transformed yeast grow up, we validate them by colony PCR,you can find the protoco for colony PCR in our wiki.

5）After we validate the germ is right, we measure the fluorescence intensity by microplate reader to compare three different reporter genes.

Week nine:2018-09-03 to 2018-09-09

Constructing KaiABC plasmids of the KaiB-KaiC combination

1)After we have successfully obtained the fragments, we design to transform ten fragments into one cell. So, we do this experiment as the following protocol:

2)First, we have to obtaining three of fragments with homology arms. Instruct by the following protocol:

Volume of 50μL

Phanta max buffer(2X)	25μL
primers	2μL each
template	1μL
dNTP mix(10mM)	1μL
Phanta max super-fidelity DNA polymerase	1μL

The PCR program are as followed:
95℃ 3min
95℃ 15s
Tm-3℃ 15s ┐ 30 cycles
72℃ 30s ┘
72℃ 5min

The gene assemble onto the pRS413 and their Tm are shown on the chart below:

TDH3P	56℃
BD	57℃
KaiC	56℃
ADH1T	54℃
PGK1P	50℃
RpaA	54℃
PGK1T	56℃
TEF1P	50℃
CikA	54℃
TEF1T	56℃

The gene assemble onto the pRS413 and their Tm are shown on the chart below:

TDH3P	56℃
AD	57℃
KaiB	54℃
ADH1T	54℃
PGK1P	50℃
KaiA	54℃
PGK1T	56℃
TEF1P	50℃
SasA	53℃
TEF1T	56℃

After PCR, we have to electrophoresis the produce in the 1% agarose to validate it. Then we take back the DNA by validate it using the kit.

3)After we obtain the purified DNA,we have to transform those into the yeast cell together with the pRS413,pRS415 and pRS416 plasmids. We choose the Yeast homologous recombination method to do this. We transform the fragments into yeast.you can find the yeast transformation's protocol in our wiki.

4)When the transformed yeast grow up, we validate them by colony PCR,you can find the protocol in our wiki.

Week ten:2018-09-10 to 2018-09-16

1) If our validation result shows that our strain is right, then we extract the plasmid out of the cell and transform them into escherichia coli, and smear them onto the solid media.

2) After the germ grow up, we select some of them to do the colony PCR, if the result is right, then we extract the plasmids out and transform all of three different plasmids in to the yeast cell.

3)After the yeast grow up,we have to do the colony PCR to test whether the strains are right.

4)After we have successfully design the strain, we have to measure the fluorescence intensity to detect whether the system we design have vibrated.

Week eleven:2018-09-17 to 2018-09-23

Deletion of ISWI,ISWⅡ

Assemble two gRNA fragments with pxzx400 plasmid,instruct by the following protocol:

1)Adhesion to gain gRNA

Volume of 20μL

oiHB063(100mM)	9μL
oiHB064(100mM)	9μL
10×Annealing buffer	2μL

The PCR procedure:

95℃ 5min

90℃ 15s ┐

├ Minus 5 degrees for each cycle，14cycles.

90℃ 15s ┘

20℃ ∞

2)We inoculate the bacteria in the culture medium to let it grow. When they After reproducing to the right concentration, we extract the plasmid pxzx400 out of the cell.

3)To get the linearized plasmid,we use the NotI and enzyme to cut the plasmid. The protocol is as followed.

plasmid	70μL
10×cutsmart	8.1μL
NotI	2.5μL

37℃ water bath 90min.

4)After the water bath, we get the linearized plasmid. To test whether the linearized plasmid is right, we have to ionophoresis the sample in the 1% agarose gel. If the length is right, cut that part of gal and purify it to obtain the right linearized plasmid.

5)Construct the gRNA and the linearized plasmid - Gibson Assembly

The protocol is as follow:

5×SE cloning buffer	2μL
gRNA	0.5μL
pxzx400	6.5μL
SE Recombinase	1μL
total	10μL

Place the mixture in the 37°C incubator for 1 hour.

Notice:The products that Gibson assembled needed to be used at once, can not be long reset.

Week twelve:2018-09-24 to 2018-09-30

1)After the Gibson assembly, we got the integrated plasmid. Then we transform it to the colibacillus to amplify the plasmid.

2)When the colibacillus grow into colonies, select four colonies smear to the Sc-URA

solid medium.
3) After the colibacillus grows to a certain density, we mix the cell with the Sc-ura liquid medium, waiting for it to grow in to a certain density, then we can extract the plasmid out of the colibacillus and send it to sequencing.
4)According to the sequencing result, we choose a right strain of pxzx400, then mix the colibacillus in the 50% glycerinum and store it in the -80°C refrigerator.
5)Using three PCR reaction systems to obtain the fragments with homology arms.
The system is as below:

ddH2O	8.5μL
2×phanta Max Buffer	12.5μL
dNTP Mix(10mM)	0.5μL
Upstream primer	1μL
Downstream primer	1μL
phanta Max Super-Fidelity DNA polymerase	0.5μL
Template DNA	1μL

6)After pcr, we fetch 10μL sample mixed with 2μL loading buffer, agarose gel electrophoresis to examine whether the length is right.
7)We fetch 50μL sample to do the overlap PCR. Take each of the three mixed systems obtained in step (1) as the template, a total of 3μL, or the mixed system obtained in step (3) as the template, and take the sample of 1μL, p 50μL large sample.
The system of the overlap PCR is as below:

ddH2O	16μL
2×phanta Max Buffer	25μL
dNTP Mix(10mM)	1μL
Upstream primer	2μL
Downstream primer	2μL
phanta Max Super-Fidelity DNA polymerase	1μL
Template DNA	3μL

After overlap PCR, we obtain the produce mixture. So we have to purify it by agarose gel electrophoresis.

Week thirteen:2018-10-01 to 2018-10-07

1)Transformation of yeast: Transform the plasmid with gRNA into the strain whose ISWI,ISWⅡ genes has been deleted, whose plasmid with ura label has been lost, but still has the plasmid with Cas9 in.

Notice: This time, we set the control group as transforming the plasmid labeled with gRNA into BY4741. And another way is transforming the plasmids labeled with gRNA and Cas9 into BY4741.
2)When the yeast grow up on the culture medium, select 92 single colonies and culture by the streak method on the solid media. Culture in 30°C incubator overnight. (The remaining four wells on the 96-well plate were reserved for the control group.)

3)PCR Validation: Colony PCR

Boil the cell in 20mM NaOH (50μL) with the following program.

99℃ 5min
4℃ 1min
} 3cycles

Then use the boiled cell as the template, prepare the solution system as followed:

template	1μL
Upstream primer	0.3μL
Downstream primer	0.3μL
10×TransFast Taq Buffer	1.5μL
2.5mM dNTPs	1.2μL
TransFast Taq DNA Polymerase	0.3μL
ddH2O	10.4μL

Do the PCR validation with the following program:

94℃ 3min
94℃ 5s
Tm-3℃ 15s
72℃ 30s
} 30 cycles
72℃ 7min

Then select the right single colony and culture by the streak method on a new solid media for future validation.

4)When the cell grown up, select the single colony and culture by streak method on the solid media. When that cell grows up, validate them by colony PCR. Using three pairs of primers to validate whether the ISWI,ISWⅡ and ISWI,ISWⅡ gene have been deleted. After obtaining the right strain, lost the plasmid and store it with glycerinum method at -80°C.