Notes of Protein Engineering

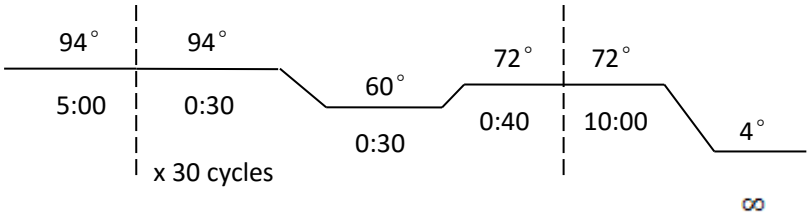
Week 2 (01/ 08/ 2016-07/ 08/ 2016)

Aug 5th

1. PCR Verify that the E.coli transformed with pRS416 linked with parts is well constructed.

   Fast Taq Polymerase 50μl Protocol;

   Strategy: Tm=60℃



   Result: Failed

2. PCR Promoter PGK1 from *Saccharomyces cerevisiae* genome

   Pfu polymerase 50μl protocol;

   Strategy: Tm=48°-43° Touchdown 0.2°/cycle x25 cycles; maintain 43° x10 cycles

   Result: Succeed

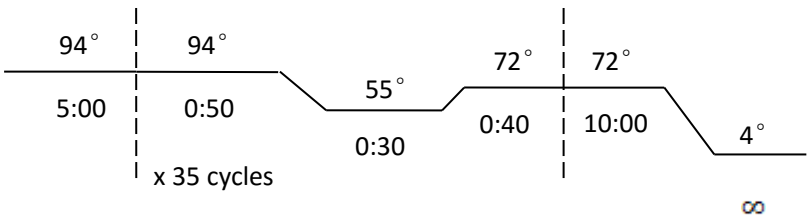
Aug 6th

1. Extracted pRS416 constructed with target genes from E.coli I II III transformed before.

   PCR Verify

   Fast Taq Polymerase 50μl Protocol;

   Strategy: Tm=55℃



   Result: Plasmids in single colony I and II have distinct target 750bp bands. The colony III does not.

2. Cut the pRS416 above with enzyme SacI and SalI to cut off PETase to check if it's linked on the plasmid or not by agarose gel electrophoresis

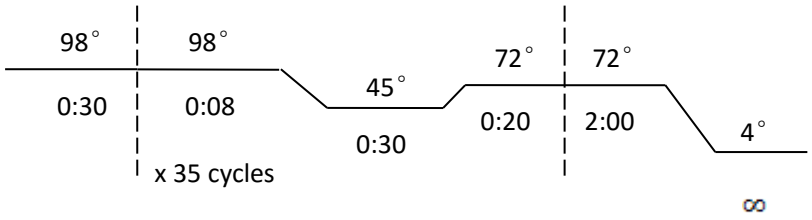
   50µl Protocol

   Result: Failed. No 750bp target band, which is confusing since the PCR Verify worked.

3. PCR PGK1 from S.c. yeast genome

   Q5 polymerase 50µl protocol;

   Strategy: Tm=45℃



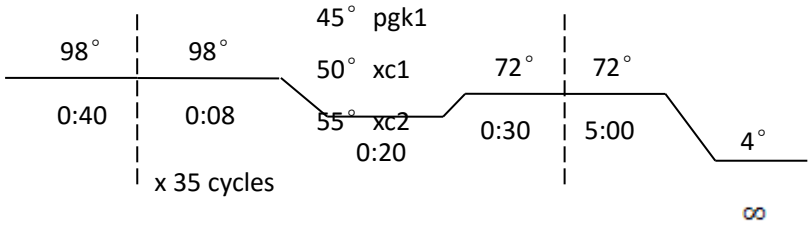
   Result: Failed

Aug 7th

1. PCR:PGK1

   Q5 polymerase 50µl protocol;

   Strategy: Tm=45℃ Tm=50℃ Tm=55℃

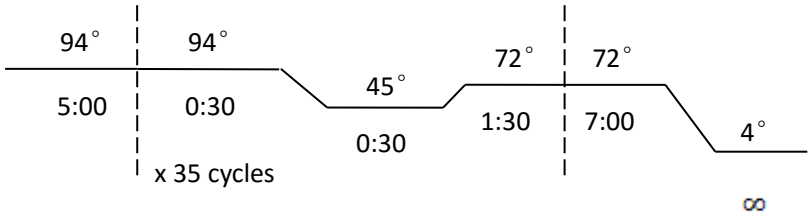


   Result: Succeeded

3. As the verifying result of PCR and enzyme cut is antithetical, we performed another PCR whole 4 parts from PGK1 to CYC1(with the s-primer of PGK1 and a-primer of CYC1) to confirm if the parts are linked on pRS416 or not.

   Fast Taq Polymerase 50µl Protocol;

   Strategy: Tm=45℃



Result: Succeeded with obvious 2k target band.

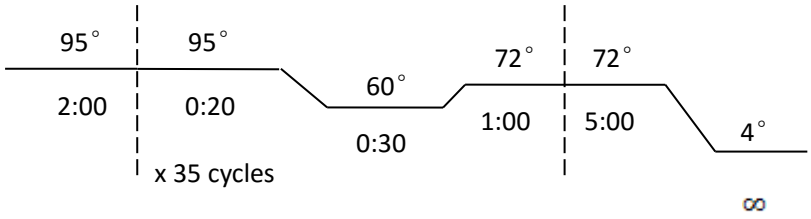
Week 3 (08/ 08/ 2016-14/ 08/ 2016)

Aug 8th

1. Re-Overlaped all Mutant fragments with primers with Not I & EcoR I Restriction enzyme site, used for constructing a new vector.

   Pfu Polymerase 50μl Protocol;

   Strategy: Tm=60℃



Result: Succeed and recycled them from the agarose gel.

Aug 9th

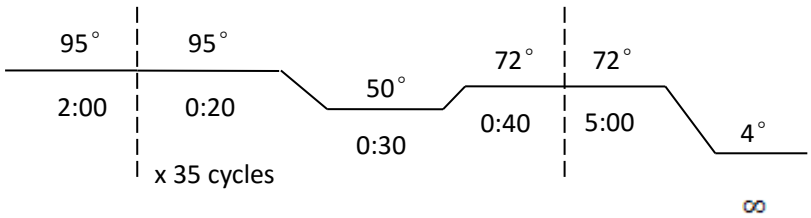
1. Cut the pRS416 above to check if the parts are linked with restriction enzyme site Sal I & Sac I, and BamH I & EcoR I respectively with a 50μl Protocol for 2 hours.

   Result: Failed.

2. PCR: α-factor signal peptide with primer with Not I Restriction enzyme site

   Pfu Polymerase 50μl Protocol;

   Strategy: Tm=50℃



Result: Succeed

Aug 10th

1. Extract unloaded plasmids pRS413 & pRS415 to construct a new expression vector.
2. Cut plasmid pRS415 with restriction enzyme Sal I & Sac I in a 50μl protocol and checked by agarose gel electrophoresis with a positive control (uncut plasmid pRS415)

Result: Succeeded. The enzyme cut worked.

3. Cut 4 parts PGK1, a-factor peptide, PETase Mutant and CYC1 respectively with the following restriction enzymes in 50μl protocols.

PGK1: SacI & BamHI

α-factor: BamHI & NotI

PETase Mutant: NotI & EcoRI

CYC1: EcoRI & SalI

And the enzyme cut products are respectively purified and tested the concentration.

3. Ligation of cut pRS415 and four cut parts above with T4 ligase under 22° for an hour in a 50μl protocol. Meanwhile, we linked only pRS415 without 4 parts above in a 10μl protocol as negative control.
4. Transformed the product and negative control of ligation above into E.coli

   Result: Failed. No colony grew.

Aug 11st

1.Cut all the PETase Mutation fragments with Not I & EcoR I restriction enzyme in a 50 protocol for further ligation. Purified and tested the concentration of the products of enzyme cut.

2.Ligation of 4 parts and cut pRS415 with T4 ligase under 22° for 2 hours and 3 quarters as well as a negative control.

3. Transformed the products above into E.coli.

Aug 12nd

1. One of the transformations yesterday succeeded. One colony of mutant I208V successfully survive on the plate. We cultured the colony at 30° and 220 rpm for 8 hours.

   Discussion: We assumed the efficiency of ligation of four parts and plasmid at one

time is rather low. That's why only one single colony succeeded.

2.Extracted the linked plasmid pRS415 above and tested its concentration.

3.PCR verify the linked pRS415 extracted above if the ligation is actually successful in the three systems as follows.

• PCR from PGK1 to CYC1 with forward primer of PGK1 & reverse CYC1 primer and Tm=45°

•PCR from α-factor to PETase Mutant I208V with former primer of α-factor & reverse PETase primer and Tm=50°

• PCR PETase Mutant I208V with PETase forward and reverse primers and Tm=60°

All the PCR above were performed in FastTaq 20μl protocol.

The products of enzyme cut are checked by agarose gel electrophoresis with a negative control of uncut pRS415 loaded with the four parts as well as a positive control of a cut pRS415.

Result: Succeeded. The three bands are respectively of 2k length, 1k length & 750bp length as expected. Though the bands aren't quite obvious and the cut plasmid is around 5k.

5. Cut the linked pRS415 extracted above with restriction enzyme Not I & EcoR I to check if the ligation succeeded and to cut off the PETase mutation I208V fragment to gain the pRS415 backbone with PGK1-α-factor and CYC1 on and substitute with other mutation site fragments. Checked by agarose gel electrophoresis.

Result: There is a obvious bright band of 5k, which is confusing since the pRS415-PGK1-α-factor-CYC1 band is supposed to be around 7k. But it can be verified the cut worked.

Aug 13rd

1. Recycled the cut pRS415 backbone yesterday from agarose gel

2.Check the backbone by agarose gel electrophoresis with a negative control and a positive control.

Result: The positive control is a 6k band as expected. However, the backbone band is 3.5k, which is supposed to be 7k.

Discussion: After double-check of the map of pRS415, we found another EcoR I restriction enzyme site locating in Leu, whose cut product is exactly 3.5k. In this way, we can't use the backbone directly substituted with different mutations. So we, again set on the way to construct another backbone.

3.Cut pYES2(from Invitrogen) with restriction enzyme BamH I & EcoR I in a 50μl protocol for 2h. And recycled the product grom agarose gel and tested the concentration.

4.Ligation of cut pYES2 above, 22 PETase Mutants and α-factor peptide respectively in a 20μl protocol under 22° for 3 hours.

5.Transformed the products of ligation above into Saccharomyces cerevisiae.

6.We tried to make the most of the linked pRS415 vector which is well constructed but not capable of being cut into a backbone. So we cut off the segment of the linked four parts, PGK1-α-factor-PETaseMutant-CYC1. Checked by agarose gel electrophoresis and recycled from agarose gel.

Result: Extremely bright standard 2k band as expected.

Aug 14th

1. Cut pRS413 with Sac I and Sal I enzyme restriction site. Recycled from agarose gel.
2. Ligation of the cut pRS413 and the 2k segment of PGK1-α-factor-PETaseMutant-CYC1 in a 20μl protocol under 22° for 3 hours.
3. Transformed the product of ligation above into E.coli.

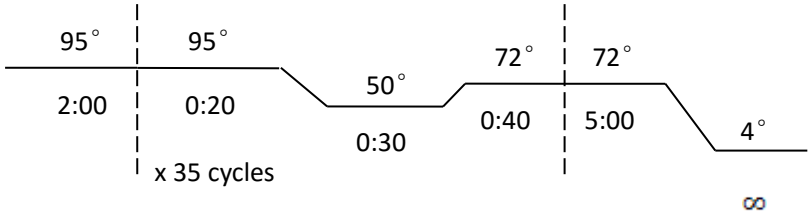
Week 4 (15 /08 /2016-21 /08 /2016)

Aug 15th

1. PCR a-signal pepitide

   Pfu Polymerase 50μl Protocol;

   Strategy: Tm=50℃



   Result: Succeed

2.Extracted plasmid blank pYES2

3.Cut pYES2 with restriction enzyme BamH I & EcoR I in a 50μl protocol and recycled the product from agarose gel.

4.Cut α-factor peptide with enzyme Not I & BamH I in a 50μl protocol and purified

the product.

5. Extracted the plasmid pRS413 linked with the 2k segment constructed yesterday.

Aug 16th

1. We found the transformation of pYES2 to yeast on 13th succeeded and some colonies in each plate have grew.

PCR Verify pRS413 extracted yesterday if the ligation is actually successful in the three systems as follows.

• PCR from PGK1 to CYC1 with forward primer of PGK1 & reverse CYC1 primer and Tm=45°

•PCR from α-factor to PETase Mutant I208V with former primer of α-factor & reverse PETase primer and Tm=50°

• PCR PETase Mutant I208V with PETase forward and reverse primers and Tm=60°

All the PCR above were performed in FastTaq 20μl protocol.

The products of enzyme cut are checked by agarose gel electrophoresis with a negative control as well as a positive control.

Result: the first and third PCR worked. There is band of 2k and 750bp. But there is no band of 1k.

2.Cut the linked pRS413 with restriction enzyme Not I & EcoR I to check if the ligation succeeded. Checked by agarose gel electrophoresis with two negative controls and a positive control.

Result: Failed. No supposed band of 750bp existed.

3.Made Sc culture medium.

4. Sent pRS413 and pYES2 constructed to sequence.

Aug 17th

1. Colony PCR verify the constructed pYES2 with 22 mutants transformed in yeast in a 20μl protocol of 6 colonies of each mutants. Tm=60°

   Result: aragose gel picture as follow.

   Some colonies succeeded and some didn't in each mutant.

2. Made Sc-Ura medium.

Week 5 (22 /08 /2016-28 /08 /2016)

Aug 22nd

1. Made Sc-Ura culture
2. Prepared 100x Leu

Aug 26th

1. Induced Gal1 promoter in the yeast transformed pYES2 constructed above.
2. Assay pNPA with the induced yeasts with 2% glycerol and 2% galactose in final concentrations of 0.5mM PNPA in PBS Buffer (pH7.4). The reaction was initiated by the addition of pNPA. The hydrolysis of pNPA was monitored by the formation of the p-nitrophenol at 400nm in a Biotec microplate reader.
   Mutants: W159M, S238F, W159H, R90T, W159A, S92A, R90I, A240P

Aug 28th

1. Another round of induction of Gal1 Promoter.
2. Measurement of the induction of the Gal1 promoter with galactose
   Six different kinds of 5 mL cultures of saccharomyces cerevisiae, harboring plasmid S207T, R90A, 208, W159A, 238, A240P respectively and Gal1 promoter, were cultivated overnight in Sc-Ura culture medium with glucose at 37 ℃.
   The six 5 mL overnight cultures were centrifuged in 4000rpm for 5 min and inoculated to 5 mL Sc-Ura culture medium without glucose, for the purpose of running out of glucose inside the cultures.
   After 4 h, the six cultures were centrifuged in 4000rpm for 5 min and inoculated into six 5 mL cultures with galactose.
   A culture without galactose was used as control and 208 was inoculated into it.
   Culturing at 37℃.
   After 2 h, 100 μL per culture were transferred into a 96 well plate, 100μL pNPA solution was added into each well and measured. Each culture has 3 parallel controls. The 208 culture and pNPA solution were also measured as control.
   The 7 cultures were cultivated continuously.
   After 1h, 5000μL pNPA solution was added into each culture.
   After 2h, 200μL per culture were transferred into a 96 well plate. Each culture has 3 parallel controls.

Week 6 (29/08/2016-04/09/2016)

1. Another round of induction of Gal1 Promoter.
2. Measurement of the induction of the Gal1 promoter with galactose

Six different kinds of 5 mL cultures of saccharomyces cerevisiae, harboring plasmid S207T, R90A, 208, W159A, 238, A240P respectively and Gal1 promoter, were cultivated overnight in Sc-Ura culture medium with glucose at 37 ℃.

The six 5 mL overnight cultures were centrifuged in 4000rpm for 5 min and inoculated to 5 mL Sc-Ura culture medium without glucose, for the purpose of running out of glucose inside the cultures.

After 4 h, the six cultures were centrifuged in 4000rpm for 5 min and inoculated into six 5 mL cultures with galactose.

A culture without galactose was used as control and 208 was inoculated into it.

Culturing at 37℃.

After 2 h, 100 μL per culture were transferred into a 96 well plate, 100μL pNPA solution was added into each well and measured. Each culture has 3 parallel controls. The 208 culture and pNPA solution were also measured as control.

The 7 cultures were cultivated continuously.

After 1h, 5000μL pNPA solution was added into each culture.

After 2h, 200μL per culture were transferred into a 96 well plate. Each culture has 3 parallel controls.

3. Repeat Step 1 and 2.