Molecular cloning: Pcat-2M-lacI/tetR-term+lacP/tetP

Parts: K228815/16+R0010/R0040=K228817/18

Resource:

Pcat-2m-lacI/tetR-term (K228815/16): myself, plasmid, rename as L, T.
lacP: part R0010, from He Siheng, already digested;
tetP: part R0040, myself, already digested (July 20th)

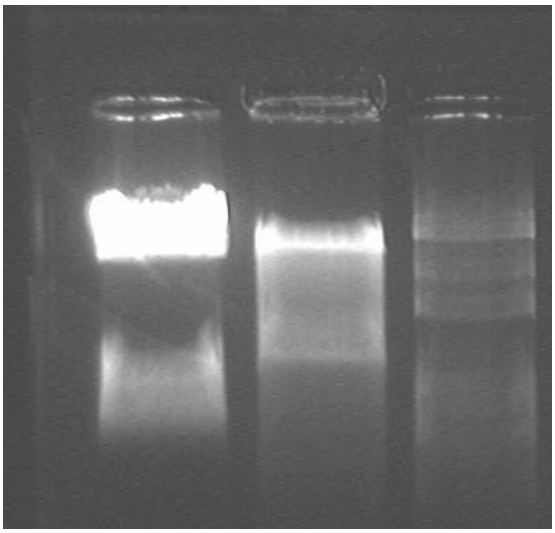
July30 th

Double digest:

L, T: Spe1 1uL, Pst1 1uL, plasmid 10uL, Buffer 2uL, water 6uL
37 ℃ 4 hour

Gel electrophoresis:

Products of double digest of L, T
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 30min
lane1: digested product of T;
lane2: digested product of L;
lane3: marker;



The insert of T is about 900bp.
The insert of L is about 1.4kb.

DNA Gel purification:

Inserts of L, T.

DNA ligation:

System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL

16℃ overnight.
Insert: T *2;
Vertor: tetP (has already digested by EcoR1 & Xba1)

July 31st

Transformation:
Products of ligation (T+tetP *2), competent cells 50uL each,
Smear to LB plate with Amp

DNA ligation:
System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL
16℃ overnight.
Insert: L *2;
Vertor: lacP (has already digested by EcoR1 & Xba1, by He Siheng)

Transformation:
Products of ligation (L+lacP *2), competent cells 50uL each,
Smear to LB plate with Amp

August 1st
Every plate is very well: more than 100 clones

PCR: (colony PCR, T-tetP)
System 10 uL: Master mix 5ul, primer (standard primer) 0.5uL each, water: 4uL template; 10 colonies of T-tetP;
Gel electrophoresis: (help by Lin Min)
Refer to Lin Min's notes,
All of 10 colonies are wrong!!!
Repeat!!!

Double digest: (again, tetP)
tetP: EcoR1 1uL, Xba1 1uL, plasmid 4uL, Buffer 2uL, water 12uL
37 ℃ 4 hour

Transfer colonies: (L-lacP)
Transfer 6 colonies (L-lacP) into 5ml LB, and amplify the Ecoli.

Plasmid mini prep: (L-lacP)
6 colonies of L-lacP

Double digest: (to check the correct L-lacP)
6 L-lacP: EcoR1 1uL, Pst1 1uL, plasmid 4uL, Buffer 2uL, water 12uL
37 ℃ overnight

August 2nd

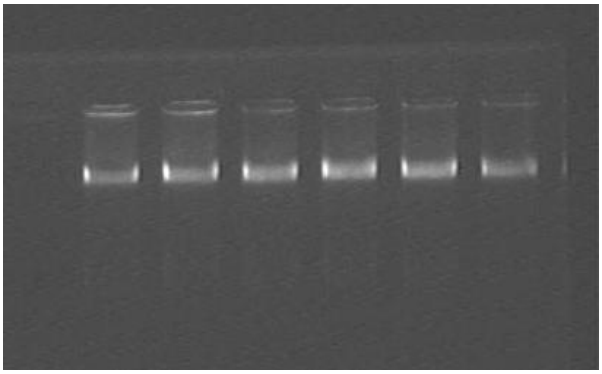
Gel electrophoresis: (check the correct L-lacP)

Products of double digest

Marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb

loading buffer and DNA dye: 6×

Voltage and time: 60V 5min; 120V 15min



I forgot to add the Marker, but from the result we can easily find that all these 6 colonies are wrong!!!

PCR: (colony PCR, L-lacP)

System 10 uL: Master mix 5ul, primer (standard primer) 0.5uL each, water: 4uL template; 24 colonies of L-lacP;

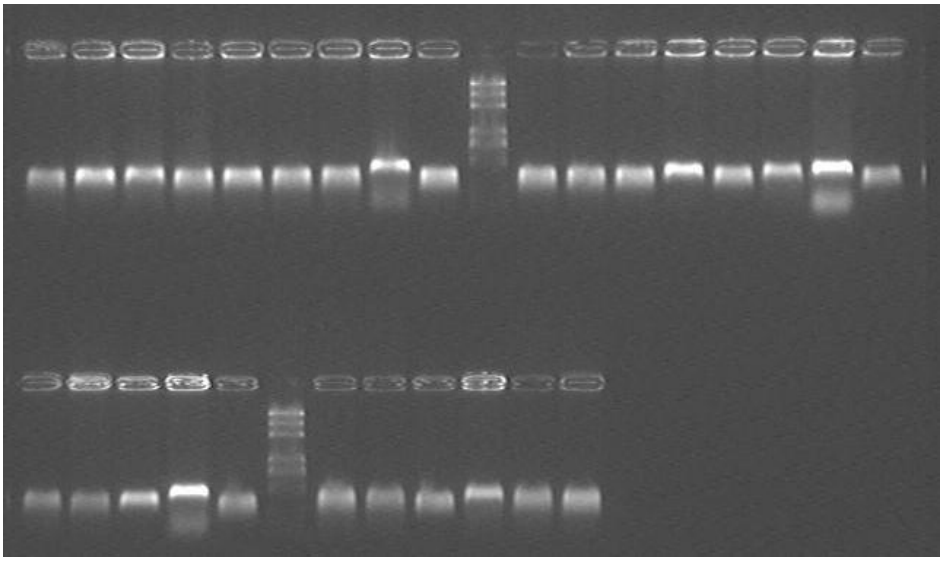
Gel electrophoresis: (check the correct L-lacP)

Products of PCR

Marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb

loading buffer and DNA dye: 6×

Voltage and time: 60V 5min; 120V 15min



From the lane 5 to the last one are 24 results of L-lacP PCR.

Since there is not any DNA larger than 1kb, all of these 24 colonies are wrong!!!

All these DNA are about 200bp. So they are the result of self link of lacP!!! There

must be something wrong with the digested lacP!!!

Double digest: (again, lacP)
lacP: EcoR1 1uL, Xba1 1uL, plasmid 4uL, Buffer 2uL, water 12uL
37 ℃ overnight!

DNA ligation (again T+tetP):
System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL
16℃ 4 hours
Insert: T *2;
Vertor: tetP (digested on Aug.1st)

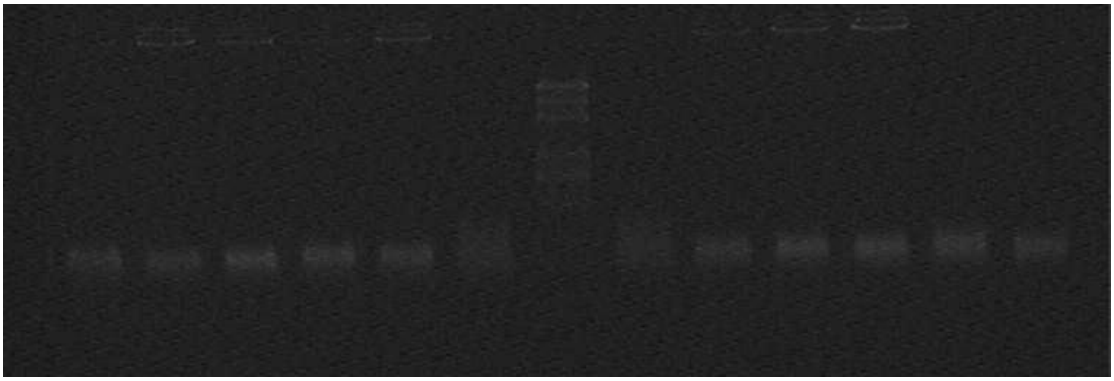
Transformation: (again T+tetP)
Products of ligation (T+tetP *2), competent cells 50uL each,
Smear to LB plate with Amp

August 3rd
PCR product purification:
lacP (digested yesterday)

DNA ligation (again L+lacP):
System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL
16℃ 4 hours
Insert: L *2;
Vertor: lacP (digested on Aug.2nd)

PCR: (colony PCR, T-tetP)
System 10 uL: Master mix 5ul, primer (standard primer) 0.5uL each, water: 4uL template; 10 colonies of T-tetP;

Gel electrophoresis:
Products of PCR
Marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
Voltage and time: 60V 5min; 120V 15min



Since there is not any DNA larger than 1kb, all of these 10 colonies are wrong

Again!!!

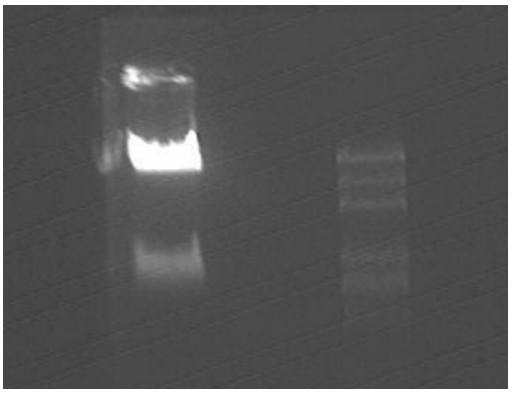
Bad Luck!!!!!

Transformation: (again L+lacP)
Products of ligation (L+ lacP *2), competent cells 50uL each,
Smear to LB plate with Amp

Double digest: (the 3rd time! tetP and T)
tetP: EcoR1 1uL, Xba1 1uL, plasmid 4uL, Buffer 2uL, water 12uL
T: pe1 1uL, Pst1 1uL, plasmid 10uL, Buffer 2uL, water 6uL
37 ℃ overnight!

August 4th
PCR product purification:
tetP (digested yesterday)

Gel electrophoresis:
Products of double digest of L, T
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 30min
lane1: digested product of T;
lane2: marker



The insert should be 900 bp, and it is correct!

DNA Gel purification:
Insert of T.

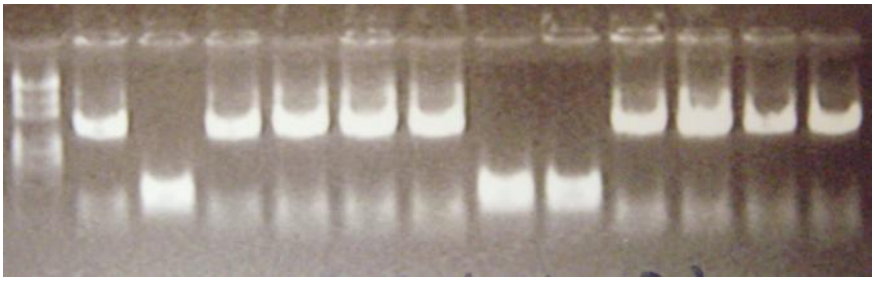
DNA ligation (the 3rd time T+tetP):
System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL

16℃ 4 hours
Insert: T *2 (new);
Vertor: tetP (new);

Transformation: (the 3rd time T+tetP)
Products of ligation (T+tetP *2), competent cells 50uL each,
Smear to LB plate with Amp

PCR: (the 2nd time colony PCR, L-lacP)
System 10 uL: Master mix 5ul, primer (standard primer) 0.5uL each, water: 4uL template; 12 colonies of L-lacP;

Gel electrophoresis: (check the correct L-lacP)
Products of PCR
Marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
Voltage and time: 60V 5min; 120V 15min



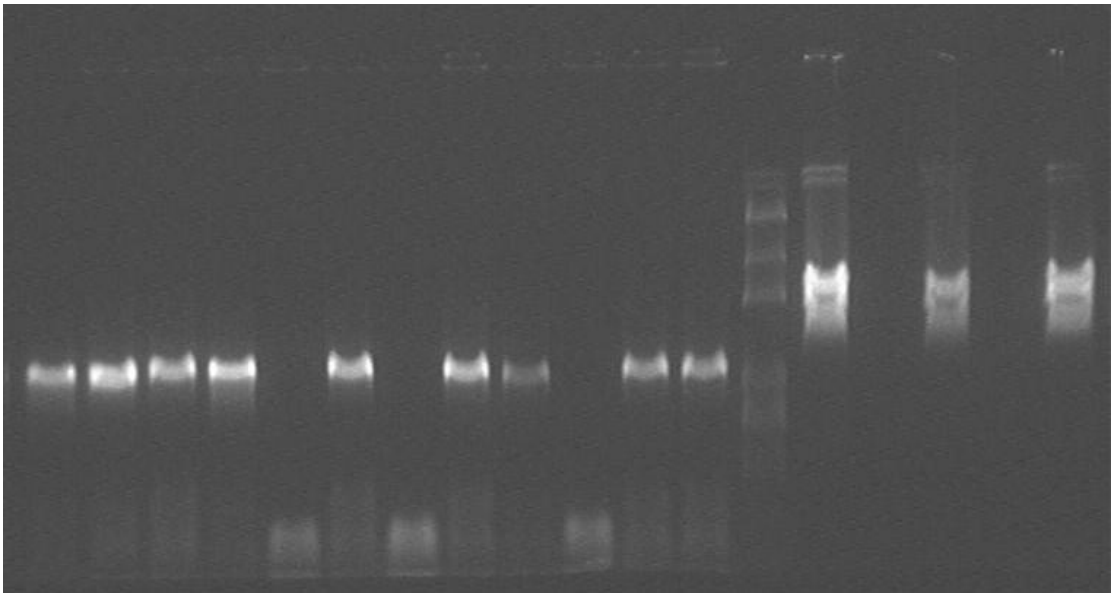
The insert of correct L-lacP is about 1.4kb!!!

9 of 12 colonies are correct!!!!!

August 5th
PCR: (the 3rd time colony PCR, T-tetP)
System 10 uL: Master mix 5ul, primer (standard primer) 0.5uL each, water: 4uL template; 12 colonies of T-tetP;

Gel electrophoresis: (check the correct T-tetP)
Products of PCR
Marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
Voltage and time: 60V 5min; 120V 60min
Lane 1~12: T-tetP 1~12
Lane 13: Marker

The insert is about 1kb, and 9 of these 12 colonies are CORRECT!!!!



Result:

At last, I successfully constructed: Pcat-2M-lacI/tetR-term+lacP/tetP, and they are the parts K228817/18.

Experience:
The vector is very important in this cloning. We should digest completely all the vectors, in order to prevent the self-linkage of the vectors. My experience is that if you want to digest 4ul plasmid as vector, you had batter digest it overnight. If you want to quick such as in two hours, reduce the amount of plasmid.

BY Shuke Wu

Molecular cloning: Pcat-2M-lacI/tetR-term-lacP/tetP+GFP

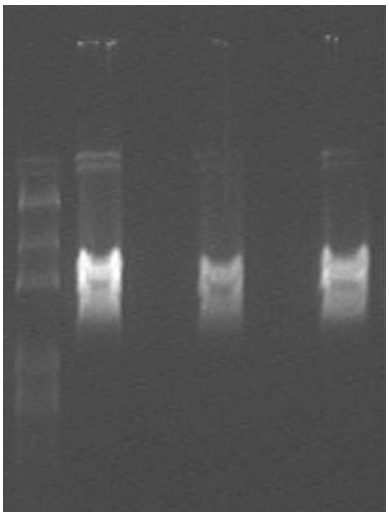
Parts: K228817/18+E0840=K228819/20

Resource:
Pcat-2M-lacI/tetR-term-lacP/tetP (K228817/18): myself, colonies, renamed as L1, L2, L3, T1, T2 and T3.
GFP (E0840): from Lin Min, vector (has already digested by EcoR1 & Xba1)

August 5th
Plasmid mini prep:
L1, L2, L3

Double digest:
L1, L2 and L3: EcoR1 1uL, Spe1 1uL, plasmid 10uL, Buffer 2uL, water 6uL
37 ℃ 4 hour

Gel electrophoresis:
Products of double digest of L1, L2 and L3,
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 60min
lane1: Marker
lane2~4: L1~3;



The insert is about 1.4kb.

DNA Gel purification:
L1, L2 and L3

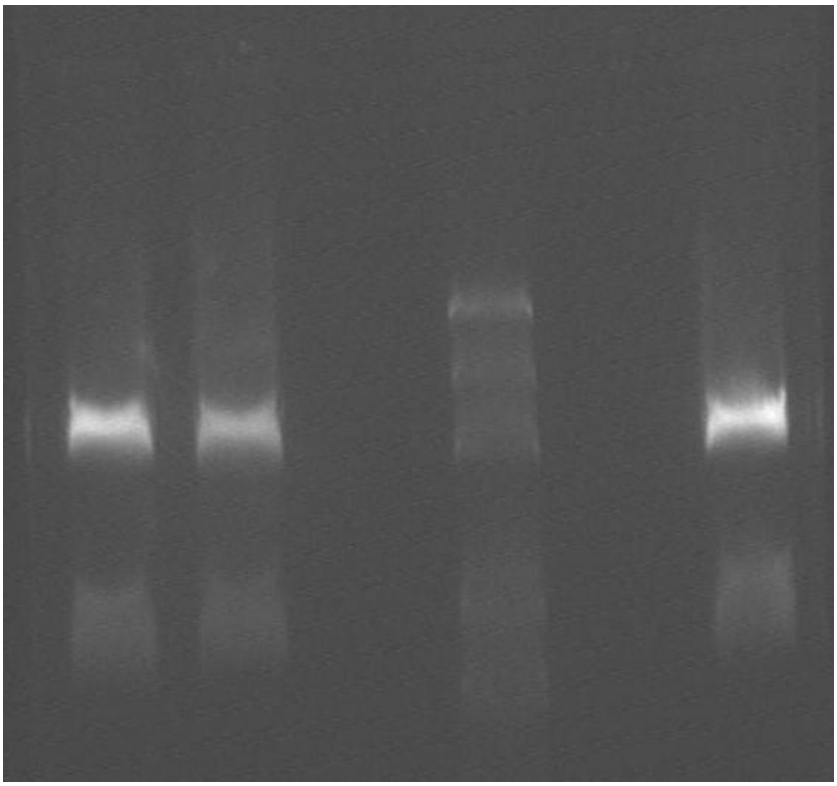
August 6th
DNA ligation:
System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL
16℃ 4 hour
Insert: L1, L2;
Vertor: GFP

Transformation:
Products of ligation, competent cells 50uL each,
Smear to LB plate with Amp

Double digest:
T1, T2 and T3: EcoR1 1uL, Spe1 1uL, plasmid 10uL, Buffer 2uL, water 6uL
37 ℃ 4 hour

Gel electrophoresis:

Products of double digest of T1, T2 and T3,
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 15min



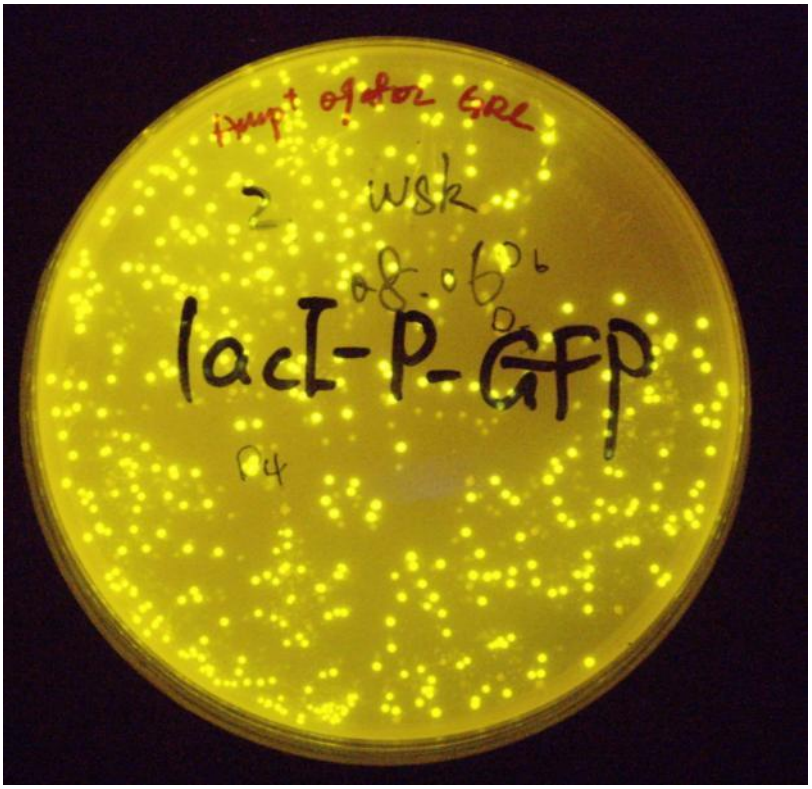
Lane 1, 2, 4: T1~3
Lane 3: Marker

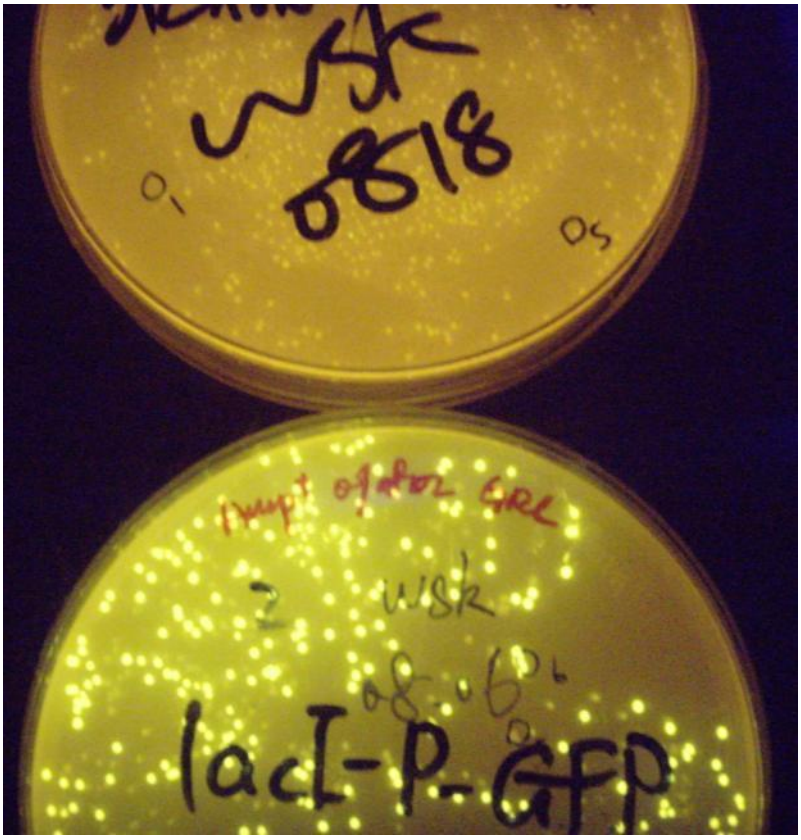
DNA Gel purification:

T1, T2 and T3

August 7th

Every plate (L1,L2 +GFP) is very well: more than 100 clones
And many colonies are become green under the blue light, which means that the expression of LacI can not fully repressed the promoter lacP.





The second picture is for comparison with no GFP colonies.

PCR: (colony PCR)

Master mix 5ul, primer (standard primer) 0.5uL each, water 4uL, template;
6 colonies of L+GFP

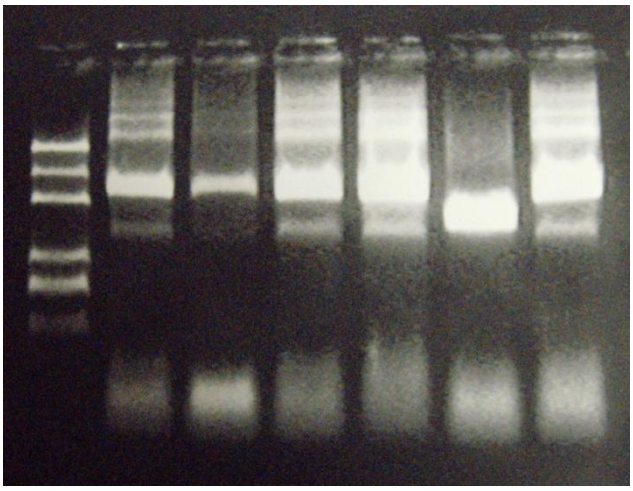
Gel electrophoresis:

Products of PCR

marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb

loading buffer and DNA dye: 6×

voltage and time: 60V 5min; 120V 15min



Lane 1: Marker;

Lane2~7: L1~6;

The correct insert is about 2.4kb, and we found that L1, L2, L3, L4, and L6 are all correct.

DNA ligation:

System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL

16℃ 4 hour

Insert: T1, T2;

Vertor: GFP

August 8th

Transformation:

Products of ligation (T1+GFP, T2+GFP), competent cells 50uL each,

Smear to LB plate with Amp

August 9th

Every plate (T1,T2 +GFP) is very well: more than 100 clones

And many colonies are become green under the blue light, which means that the expression of tetR can not fully repressed the promoter tetP.

The second picture is for comparison with no GFP colonies.

August 10th

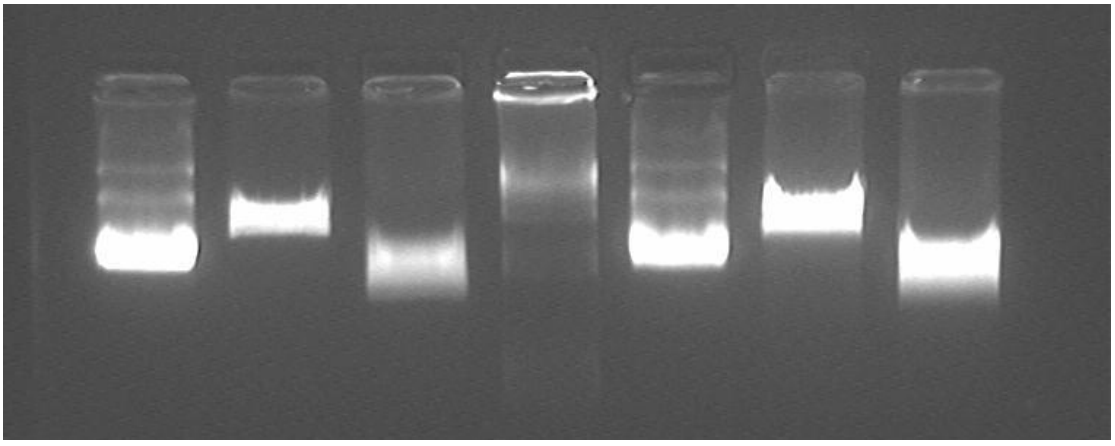
Plasmid mini prep:

T1+GFP, T2+GFP, T3+GFP;

Digest: (T1-G, T2-G)

Double: EcoR1 1uL, Pst1 1uL, plasmid 4uL, Buffer 2uL, water 12uL
Single: EcoR1 1uL, plasmid 4uL, Buffer 2uL, water 12uL
37 ℃ 4 hour

Gel electrophoresis: (to confirm the T1-G, T2-G)
Products of digest
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 15min



Lane 1 & 5: plasmid, T1-G, T2-G
Lane 2 & 6: single digest T1-G, T2-G
Lane 3 & 7: double digest T1-G, T2-G
Lane 4: Marker

The insert is about 1.8kb, and the vector is about 2.1kb. It is very hard to separate them, yet from the gel, we know that the T1-G and T2-G are correct.

Result & discussion:
I successfully constructed the two clones: Pcat-2M-lacI/tetR-term-lacP/tetP-GFP (K228819/20).
However, I disappointed to find that these clones are not work very well, because the GFP express significantly even on the plate (without induce)!!! That means the expression of lacI and tetR are not enough to repress the lacP and tetP. It is possible that the LVA tail of lacI and tetR make they degrade very soon. (for more information of LVA refer to parts C0012 and C0040). And other possibility is that the constitutive promoter Pcat is not strong enough.

BY Shuke Wu

Molecular cloning: strong promoter+Pcat-2M-lacI-term-lacP-GFP

Parts: J61100+K228819

Resource:

Pcat-2M-lacI-term-lacP-GFP: myself, colonies, renamed as L1, L2, L3.

Promoter (J61100): from Lin Min, vector (has already digested by Pst1 and Spe1)

August 8th

Plasmid mini prep:

L1, L2, L3

Double digest:

L1, L2: Xba1 1uL, Pst1 1uL, plasmid 10uL, Buffer 2uL, water 6uL

37 ℃ 4 hour

August 9th

Gel electrophoresis:

Products of double digest of L1, L2,

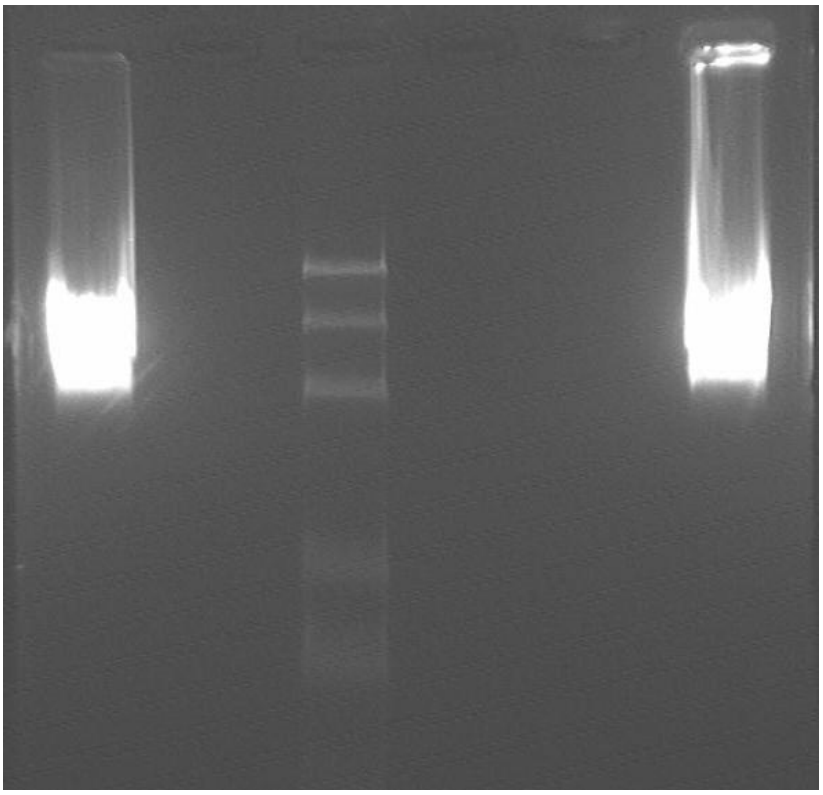
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb

loading buffer and DNA dye: 6×

voltage and time: 60V 5min; 120V 60min

lane2: Marker

lane1,3: L1, L3;



The insert is about 2.4kb, and the backbone is 2.1kb.

DNA Gel purification:
The insert of L1 and L2.

DNA ligation:
System 10uL: Insert 6uL, vector 2uL, water 3uL, buffer 1uL, T4 DNA ligase 1uL
16℃ 4 hour
Insert: L1, L2;
Vertor: strong promoter, from Lin Min.

Transformation:
Products of ligation, competent cells 50uL each,
Smear to LB plate with Amp

August 10th
Every plate (P+L1/L2) is very well: more than 100 clones
But many colonies are become green under the blue light, which means that the expression of LacI can not fully repressed the promoter lacP.

Result & discussion:
I successfully constructed clone: Promoter-Pcat-2M-lacI-term-lacP-GFP .
However, I disappointed to find that these clones are not work very well, because the GFP express significantly even on the plate (without induce)!!!
The expression of lacI is still not enough to repress the lacP.

BY Shuke Wu

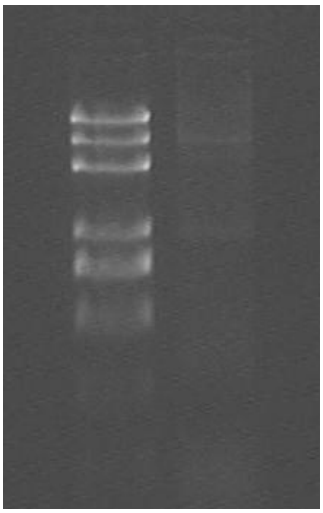
Delete LVA tail from reverse tetR-tetP-GFP

Resource:
Reverse tetR-tetP-GFP: from Lin Min, plasmid. Renamed as TPG
Reverse tetR-tetP*2, (not very confirm): from Lin Min, plasmid, Renamed as TP1, TP2
Vector: a plasmid with Kan resistant. From Lin Min
Primer:
Delete LVA primer, with complement to 20 last bps of tetR coding sequence and a TAA+Xba1 tail. And it can be use with one of standard primers (Rev), to amplify any sequence between the end of tetR and standard suffix.
5'-GCTCTAGATTAGGACCCACTTTCACATTTAA-3'
Designed by me.

August 11th
PCR: (helped by He Siheng)

System 20 uL: pfu enzyme 1ul, primer (delete LVA primer and standard primer reverse one) 1uL each, Buffer 2 uL; water 10uL; template TPG 1uL; dNTP 4uL;

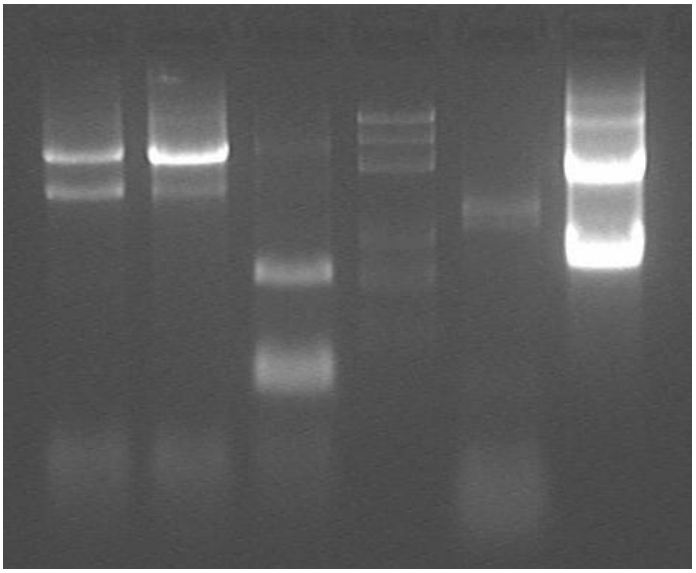
Gel electrophoresis:
Products of PCR;
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 15min
lane1: Marker;
lane2: product;



Obviously, PCR is failed!!!
Repeat!!

PCR: (repeat)
System 20 uL: pfu enzyme 1ul, primer (delete LVA primer and standard primer reverse one) 1uL each, Buffer 2 uL; water 10uL; template (TP1, TP2, TPG) 1uL; dNTP 4uL; extending time 5 min;

August 12th
Gel electrophoresis:
Products of PCR;
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 15min

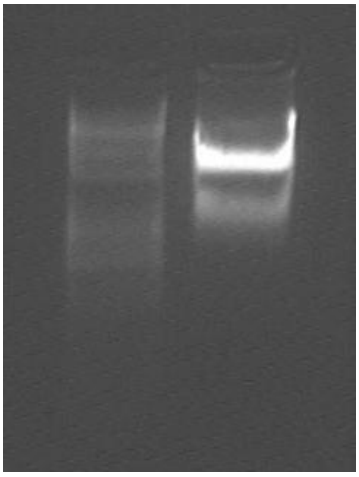


Lane1: TP1,
Lane2: TP2,
Lane3: TP1 (use another standard primer for one)
Lane4: Marker;
Lane5: negative control;
Lane6: TPG
The result is very strange:
TP1 and TP2 should be about 1kb, but there is not!
TPG should be about 2kB, but another 0.8k also very strong.

DNA Gel purification:
TPG (2kb) insert

Double digest:
TPG insert: Xba1 1uL, Pst1 1uL, DNA 16uL, Buffer 2uL,
Vector: Xba1 1uL, Pst1 1uL, plasmid 4uL, Buffer 2uL, water 12 uL,
37 ℃ 4 hour

Gel electrophoresis:
Products of digestion of vector
marker: 100bp 250bp 500bp 750bp 1kb 2kb 3kb 5kb
loading buffer and DNA dye: 6×
voltage and time: 60V 5min; 120V 15min



The vector is about 3kb.

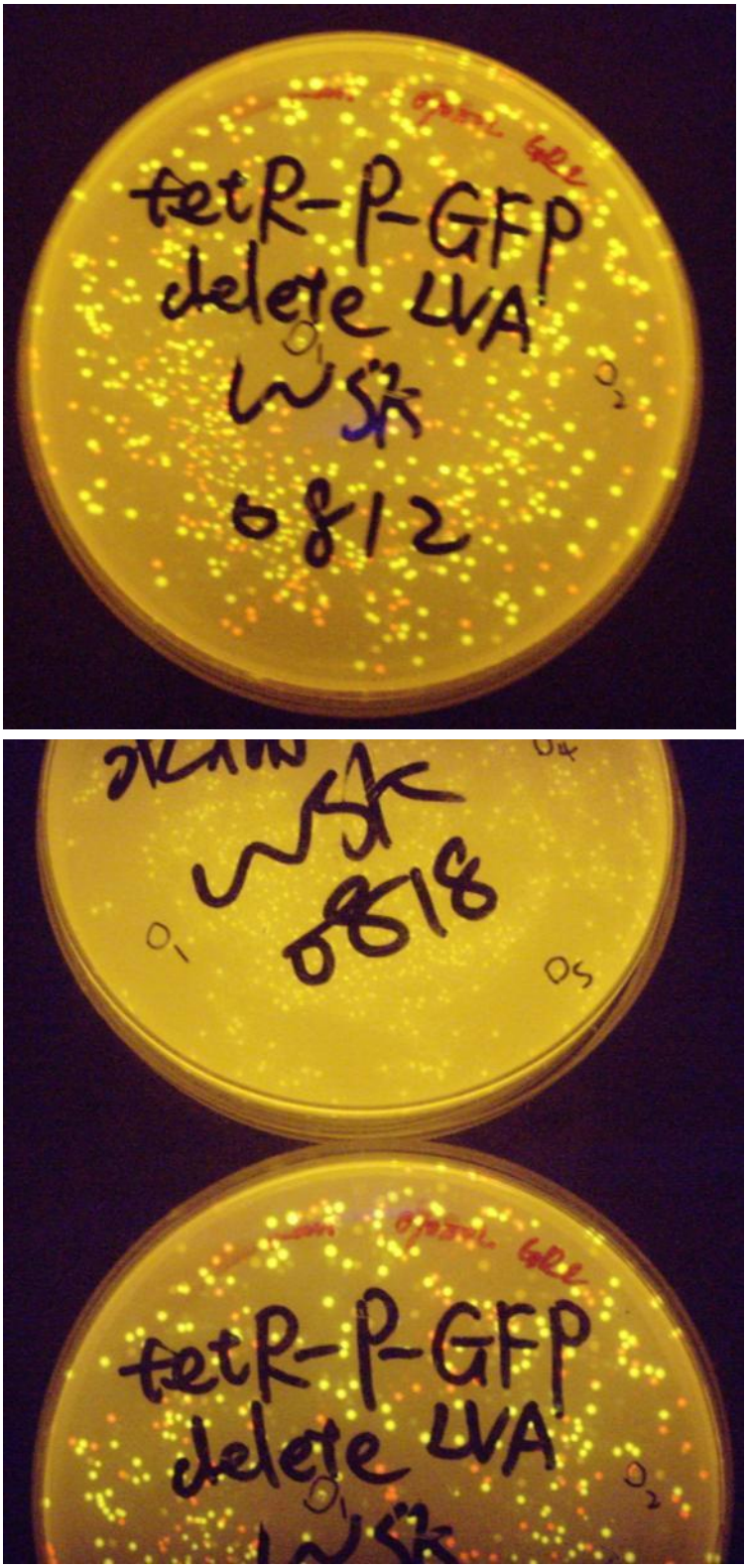
DNA Gel purification:
Vector,

PCR product purification:
Products of digestion of insert (TPG)

DNA ligation:
System 10uL: Insert 6uL, vector 2uL, buffer 1uL, T4 DNA ligase 1uL
16℃ 4 hour
Insert: TPG;
Vector

Transformation:
Products of ligation, competent cells 50uL each,
Smear to LB plate with Kan.

August 13th
The plate (delete LVA TPG) is very well: more than 100 clones
And many colonies are become green under the blue light, which means that the expression of tetR can not fully repressed the promoter tetP.
The second picture is for comparison with no GFP colonies.



August 14th

Plasmid mini prep:

Del-LVA-TPG1, 2, 3.

Result:

I successfully deleted the LVA from the Reverse tetR-tetP-GFP. Yet, the result is not very promising, because the colonies became green on the plate, without inducing.
It needs more quantitative data, but it is obviously that this cloning does not work very well.

BY Shuke Wu