Notebook for sgRNA vector construction

(written by Tanya Zhang)

Date: 9.6

1. Plasmid extraction and nucleic acid quantitative:
   - The principle:
     The plasmid is sgRNA generator.
   - The results:

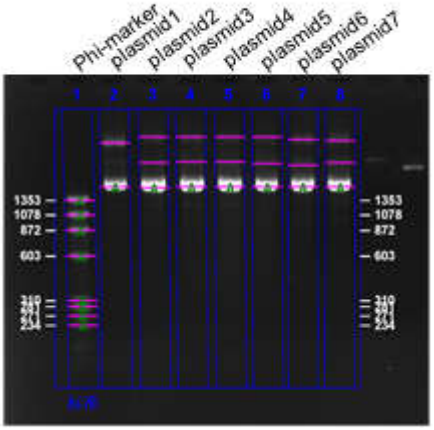

Figure1: Electrophoresis result of the original plasmid.

The concentration of the plasmid:
Tube1: 38.75ng/ul
Tube2: 64.9ng/ul
Tube3: 65.0ng/ul
Tube4: 68.9ng/ul
Tube5: 55.05ng/ul
Tube6: 61.5ng/ul
Tube7: 59.05ng/ul

Date: 9.7

1. Primer annealing:
   - The principle:
     Both primers are designed to contain the sgRNA sequence which is 20bp. The primers also contain a 4bp sequence which can base pairing with sticky end on the plasmid.
     In this experiment, the sequence of the primer GFP-sgRNA-F is TAGGCACTTTTCAAGAAGAGGAAA. The sequence of the primer GFP-sgRNA-R is AAACTTTCCTCTTCTTGAAAAGTG.
   - The system:

GFP-sgRNA-F(10mM)	5ul
GFP-sgRNA-R(10mM)	5ul
10x T4 DNA ligase buffer	2ul
ddH2O	8ul
total	20ul

   - The steps:
     - Mix the reaction system.
     - Bath the mix in water at 95℃ for 5 minutes. All primers are separated into two stands by heating to 95℃.

- Turn off the water bath. Let the temperature drop naturally to room temperature. During this process, the primers anneal.
- Store at -20℃.

2. Enzyme digestion:
   - The principle:
     We use the plasmid called sgRNA generator from Peking University. This part can be linearized by the enzyme BsaI. Then its sticky end can base pairing with the sticky end of the primer after annealing. The recombinant plasmids can be constructed.
   - The system:

BsaI	0.5ul
sgRNA generator(c=49.5ng/ul, m=0.5ug)	10ul
10x NEB buffer	2.5ul
ddH2O	12ul
total	25ul

   - The steps:
     - Mix the reaction system.
     - Incubate at 37°C for 2h.
     - Purify the fragments through gel extraction and quantify them.
   - The results:
     - The plasmids are not completely digested. There are two bands on the gel.

Figure2: Electrophoresis result of the enzyme-digested products.

- After gel extraction, the concentration of the first fragment(up) is 3.6ng/ul. The concentration of the second one(down) is 4.3ng/ul. Their concentrations are low.

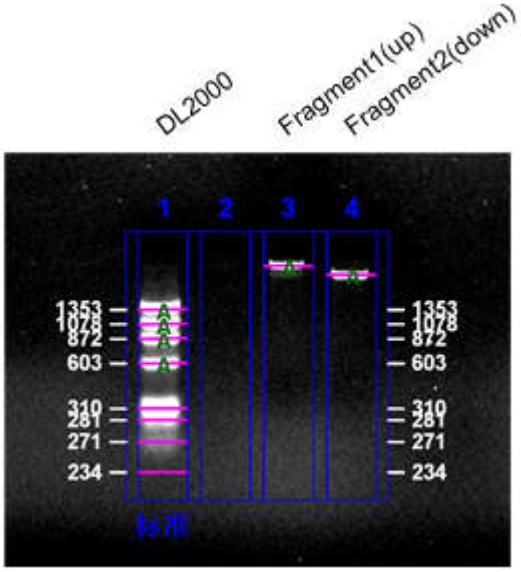

Figure3: Electrophoresis result of the two fragments which have been purified through gel extraction.

3. Ligation:
   - The system:

	The fragment above	The fragment below
The linear sgRNA generator(as mentioned above, 50ng)	13.9ul	11.6ul
The primer(2.25ng)	0.68ul	0.68ul
10x T4 ligase buffer	2ul	2ul
T4 ligase	1ul	1ul
ddH2O	2.42ul	4.72ul
all	20ul	20ul

   Note: The primer is diluted ten times in advance. And the molar ratio of the primer to the linear sgRNA generator is 5:1.
   - The steps:
     - Mix the reaction system.
     - The mix are placed at 16℃ for 8h and then at 4℃ overnight.
   - The results:
     - The results of gel electrophoresis can't accurately determine whether the fragments are connected properly. Therefore, transform the recombinant products directly.

Date: 9.8

1. Transformation:
   - The principle:
     Select the colony which may contain the correctly recombined plasmids through blue-white selection.
   - The system:

Tube1	70ul competent cells	10ul recombinant products(with the fragment above)
Tube2	70ul competent cells	10ul recombinant products(with the fragment below)
Tube3	30ul competent cells	1ul original plasmids

Tube4	30ul competent cells	0ul

- The steps:
  - Mix the reaction system.
  - Ice bath the mix for 30 minutes.
  - Heat shock the mix at 42℃ for 45s.
  - Put the mix on the ice immediately. Ice bath them for 5 minutes.
  - Add 1ml LB medium to the tube separately. And put them on the shaker at 37℃ for an hour.
  - Newcombe expetiment.
  - Note: Because X-Gal is sensitive to light, the operation should be protected from light.
  - Look at the result of transformation tomorrow.

Date: 9.9

1. Colony PCR:
   - The system:

2x T5 mix	100ul
VF2(10mM)	8ul
GFP-sgRNA-R(10mM)	8ul
Bacterial suspension	1ul
ddH2O	84ul
total	200ul

   - The steps:
     - Mark the complete single white colony.
     - Pick up the colonies with a pipette. Then pipette up and down to completely mix the bacterial suspension.
     - Mix the reaction system.
     - Set up the PCR program.

1=98.0℃ FOR 5:00
2=98.0℃ FOR 0:30
3=60.0℃ FOR 0:10
4=72℃ FOR 0:15
5=GOTO 2， 35 TIMES
6=72.0℃ FOR 2:00
7=4.0℃ FOREVER
8=END

     - Gel electrophoresis.
2. Plasmid extraction, gel electrophoresis and sequencing.
   - The results:
     There is no correct sequence.

Date: 9.11

1. Primer annealing:
    - The system and the steps are the same as before.
2. Enzyme digestion:
    - The system:

BsaI	1ul
sgRNA generator(c=55.05ng/ul, m=0.5ug)	9ul
10x NEB buffer	2.5ul
ddH2O	12.5ul
total	25ul

    Note: Compared with before, the amount of enzyme BsaI is increased to avoid incomplete digestion.
    - The steps:
        - Mix the reaction system.
        - Incubate at 37°C for 2.5h.
        - Purify the fragments through gel extraction and quantify them.

    Note: Compared with before, the time of digestion is increased to avoid incomplete digestion.
    - The results:
        - The plasmid was essentially completely digested.

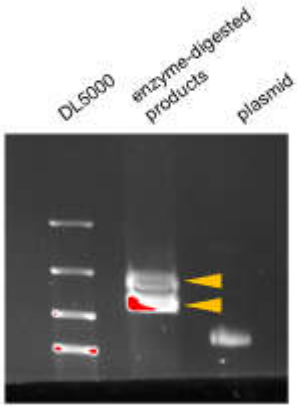

Figure4: Electrophoresis result of the two fragments which have been digested.

- After gel extraction, the concentration of the first fragment(up) is 2.35ng/ul. The concentration of the second one(down) is 6.4ng/ul.

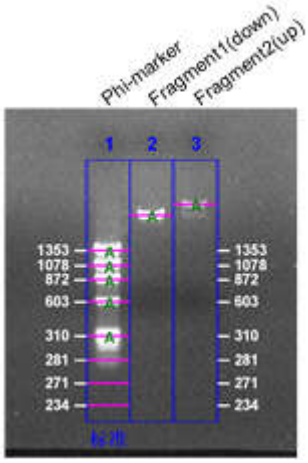

Figure5: Electrophoresis result of the two fragments which have been purified through gel extraction.

3. Ligation:

- The system:

	The fragment above	The fragment below
The linear sgRNA generator	21.28ul	7.8ul
The primer	1.02ul	0.68ul
10x T4 ligase buffer	3ul	2ul
T4 ligase	1.5ul	1ul
ddH2O	3.2ul	8.52ul
all	30ul	20ul

Note: The system of the fragment above is different. Because the concentration of the first fragment(up) is too low. Its volume has been increased.

- The steps:
  - Mix the reaction system.
  - The mix are placed at 16℃ for 8h and then at 4℃ overnight.
- The results:
  - The results of gel electrophoresis can't accurately determine whether the fragments are connected properly. Therefore, transform the recombinant products directly.

Date: 9.12

1. Transformation:
   - The system:

Tube1	80ul competent cells	10ul recombinant products(with the fragment above)
Tube2	80ul competent cells	10ul recombinant products(with the fragment below)
Tube3	20ul competent cells	1ul original plasmids
Tube4	20ul competent cells	0ul

   - The steps are the same as before.

Date: 9.13

1. Prepare the solid medium of blue-white selection.

Date: 9.14

1. Primer annealing:
   - The system and the steps are the same as before.
2. Enzyme digestion:
   - The system:

BsaI	2ul
sgRNA generator(c=58.82ng/ul, m=1ng)	17ul
10x NEB buffer	5ul
ddH2O	25ul
total	50ul

Note: Compared with before, the amount of sgRNA generator is increased. And the whole system is also changed with it. This is because the amount of the nucleic acid purified through gel

extraction is too low. This time we increase the initial amount of sgRNA generator to solve the problem.

- The steps:
  - Mix the reaction system.
  - Incubate at 37°C for 3h.
  - Purify the fragments through gel extraction and quantify them.
  - Note: Compared with before, the time of digestion is increased to achieve complete digestion.
- The results:
  - The plasmid was completely digested.

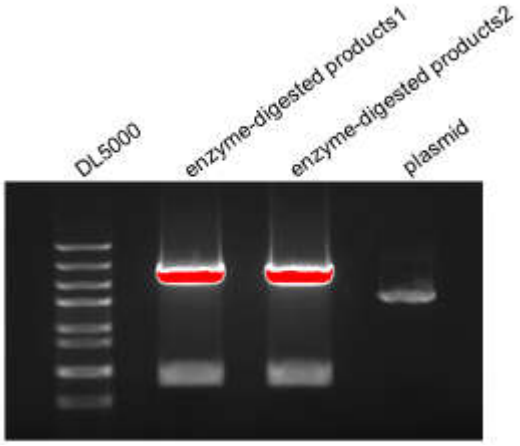

Figure6: Electrophoresis result of the two fragments which have been digested.

- After gel extraction, the concentration of the fragment is 14.1ng/ul which is great.

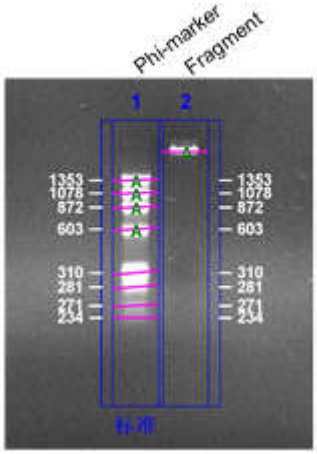

Figure7: Electrophoresis result of the two fragments which have been purified through gel extraction.

3. Ligation:
   - The system:

	The system1	The system2	The system3
The linear sgRNA generator(as mentioned above, 50ng)	3.55ul	5.92ul	7.1ul
The primer(2.25ng)	0.68ul	0.68ul	1.36ul
10x T4 ligase buffer	2ul	2ul	2ul
T4 ligase	1ul	1ul	1ul

ddH2O	12.77ul	13.4ul	8.54ul
all	20ul	20ul	20ul

Note: The system1 is the normal one. The molar ratio of the primer to the linear sgRNA generator in system2 is changed into 3:1. And the amount of nucleic acid has doubled in system3. The reason for the operation is to explore the relationship between the efficiency of the ligation and the amount of nucleic acid or the molar ratio.

- The steps:
  - Mix the reaction system.
  - The mix are placed at 16℃ for 8h and then at 4℃ overnight.
- The results:
  - The results of gel electrophoresis can't accurately determine whether the fragments are connected properly.

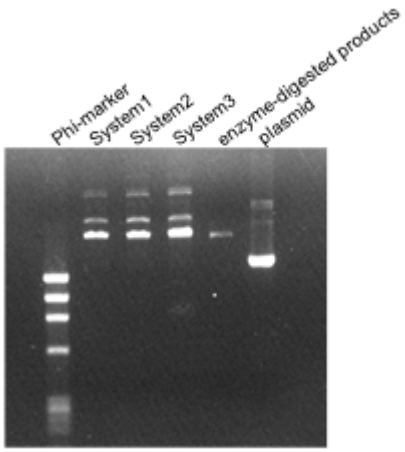

Figure8: Electrophoresis result of the connection product.

Date: 9.15

1. Transformation:
   - The system:

Tube1	50ul competent cells	2ul recombinant products(with the system1)
Tube2	70ul competent cells	10ul recombinant products(with the system1)
Tube3	70ul competent cells	10ul recombinant products(with the system2)
Tube4	70ul competent cells	10ul recombinant products(with the system3)
Tube5-PC	20ul competent cells	1ul original plasmid
Tube6-NC	20ul competent cells	0ul

   - The steps are the same as before.
   - The results:
     All three groups have large numbers of blue and white colonies.

Date: 9.19

1. Plasmid extraction and nucleic acid quantitative:
   - The principle:
     The plasmid is sgRNA generator which is prepared for follow-up experiments.
   - The results:

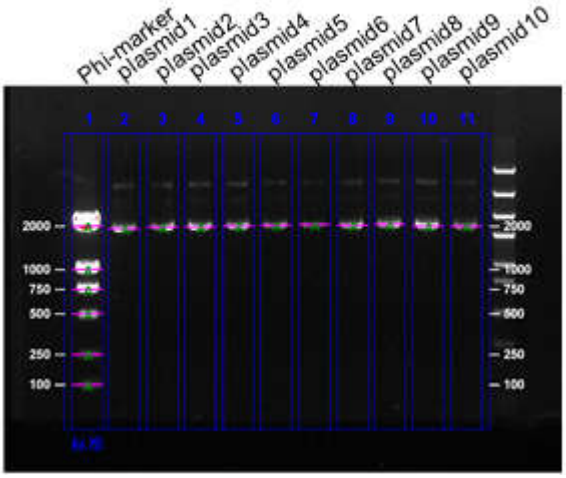

Figure9: Electrophoresis result of the original plasmid.

The concentration of the plasmid:
Tube1: 40.3ng/ul
Tube2: 37.7ng/ul
Tube3: 42.0ng/ul
Tube4: 39.2ng/ul
Tube5: 32.4ng/ul
Tube6: 27.3ng/ul
Tube7: 37.6ng/ul
Tube8: 40.6ng/ul
Tube9: 47.6ng/ul
Tube10: 33.4ng/ul

2. Colony PCR:
   - The system:

2x T5 mix	100ul
VF2(10mM)	8ul
GFP-sgRNA-R(10mM)	8ul
Bacterial suspension	1ul
ddH2O	84ul
total	200ul

   - The steps are the same as before.
   - The results:

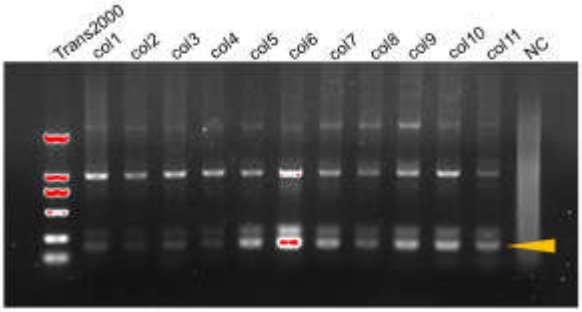

Figure10: Electrophoresis result of colony PCR. All lanes have the 140bp aim bands, while NC doesn't have the bands.

Date: 9.23

1. Plasmid extraction and sequencing.
    - The results:
      The sequencing results are correct. The recombinant plasmids are successfully constructed.
      <<COI5_VF2_TSS20180925-028-2446_B03.seq>>