Notebook for dCas9 Expression Validation and Logic Circuits Construction

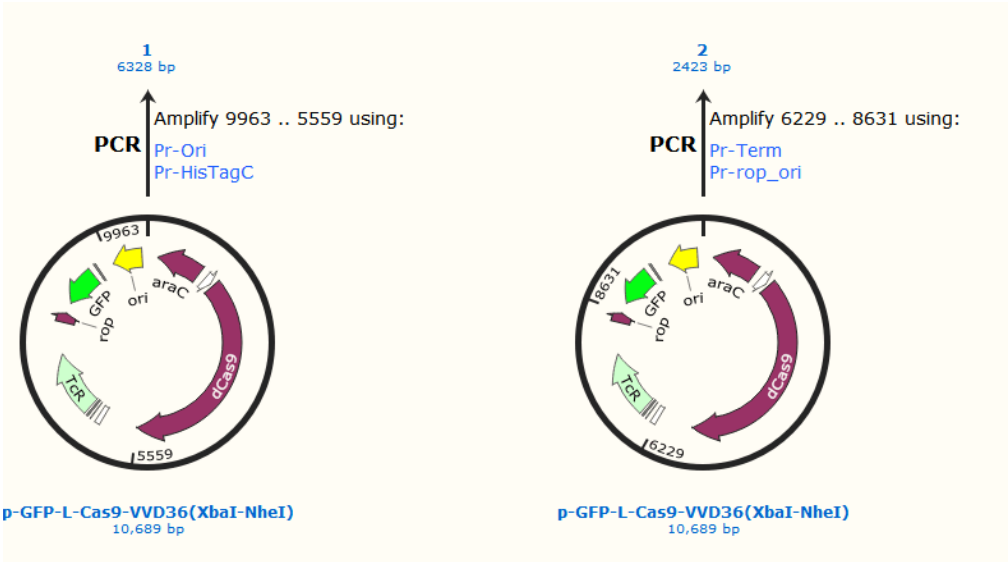
(written by Tanya Zhang)

Module 1. dCas9 expression and validation

Date: 7.23

1. Activating the glycerol bacteria with the dCas9 plasmids (pdCas9).
2. Prepare solid medium with 1/500 Tetracycline.
3. Explore the condition to amplify the two fragments on the plasmid by PCR.

- The principle:



The left one is fragment A, which contains ori, araC and dCas9. It is 6328bp.
The right one is fragment B, which contains rop and TcR. It is 2423bp.

- The system:

	Fragment A	Fragment B
2x High-Fidelity Master Mix	50ul	50ul
Primer A (10uM)	4ul	4ul
Primer B (10uM)	4ul	4ul
Template DNA (diluted 200 times)	3ul	3ul
ddH2O	39ul	39ul
Total	100ul	100ul

- The steps:
  - Measure the concentration of nucleic acids.

- The template DNA is diluted according to the measured concentration.
- Mix the system.
- Put it in the PCR machine.
- Set the thermocycling conditions to explore the optimal annealing temperature.

Steps	Temperature	Time-fragment A	Time-fragment B
Initial denaturation	98℃	2 minutes	2 minute
Denaturation	98℃ x35cycle	10 seconds	10 seconds
Annealing	53℃~57℃ -fragmentA 59℃~65℃-fragmentB	15 seconds	15 seconds
Extension	72℃	90 seconds	30 seconds
Final Extension	72℃	5 minutes	5 minutes
	4℃	Hold	Hold

- The results:
  - The concentration of nucleic acids:
    The concentration of nucleic acids is 722 ng/ul. When we use it, we dilute it 200 times.
  - The fragment A:

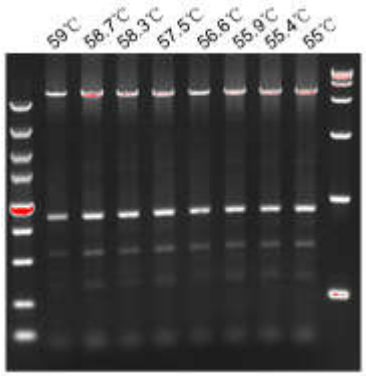


Figure: Electrophoresis result of PCR. The annealing temperature ranges from 55 to 59 ℃.

We chose 55℃ as the optimal annealing temperature.

  - The fragment B:

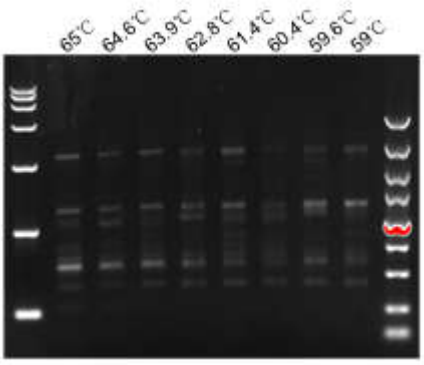


Figure: Electrophoresis result of PCR. The annealing temperature ranges from 59 to 65 ℃.

We adjust the range of the temperature to 55 to 59℃.

Date: 7.24

1. Explore the condition to amplify the fragment B by PCR.

- The system is the same as before.
- The steps:
  - Measure the concentration of nucleic acids.
  - The template DNA is diluted according to the measured concentration.
  - Mix the system.
  - Put it in the PCR machine.
  - Set the thermocycling conditions to explore the optimal annealing temperature.

Steps	Temperature	Time-fragment B
Initial denaturation	98℃	2 minute
Denaturation	98℃ x35cycle	10 seconds
Annealing	55℃~59℃-fragment B	15 seconds
Extension	72℃	30 seconds
Final Extension	72℃	5 minutes
	4℃	Hold

- The results:

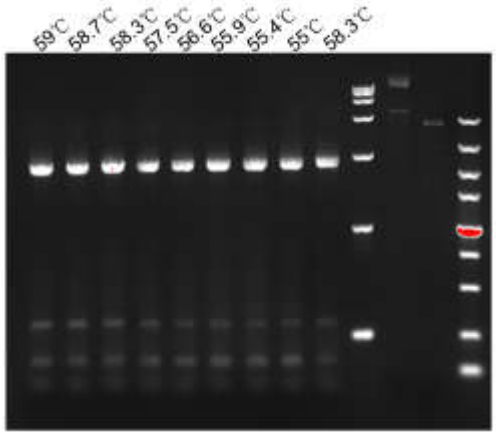


Figure: Electrophoresis result of PCR. The annealing temperature ranges from 55 to 59 ℃.

We chose the optimal annealing temperature as 58.5℃.

2. Amplify the two fragments on the plasmid by PCR.

- The system:

	Fragment A	Fragment B
2x High-Fidelity Master Mix	150ul	100ul
Primer A (10uM)	12ul	8ul
Primer B (10uM)	12ul	8ul
Template DNA (diluted 200 times)	4.5ul	3ul
ddH2O	117ul	78ul
Total	300ul	200ul

- The steps are the same as the optimal steps. The annealing temperature of fragment A is 55℃. And the annealing temperature of fragment B is 58.5℃.
- The results:

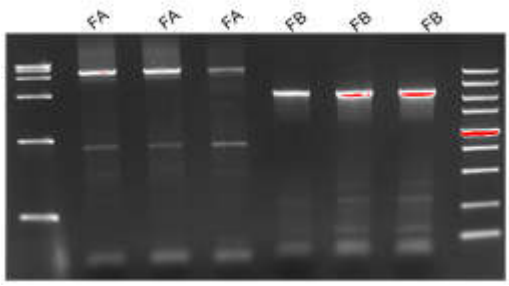


Figure: Electrophoresis result of fragment A and fragment B which are amplified by PCR.
There are non-specific bands in the figure. Therefore, we hope to adjust the reaction conditions.

Date: 7.27

1. Amplify the two fragments on the plasmid by PCR.

- The system is the same as before.
- The steps:
  - Measure the concentration of nucleic acids.
  - The template DNA is diluted according to the measured concentration.
  - Mix the system.
  - Put it in the PCR machine.
  - Set the thermocycling conditions to explore the optimal annealing temperature.

Steps	Temperature	Time-fragment A	Time-fragment B
Initial denaturation	98℃	5 minutes	5 minute
Denaturation	98℃ x35cycle	30 seconds	30 seconds
Annealing	55℃-fragmentA 58.5℃-fragmentB	30 seconds	30 seconds
Extension	72℃	180 seconds	60 seconds
Final Extension	72℃	5 minutes	5 minutes
	4℃	Hold	Hold

Note: We extend the time for each reaction to improve the reaction results.

- The results:

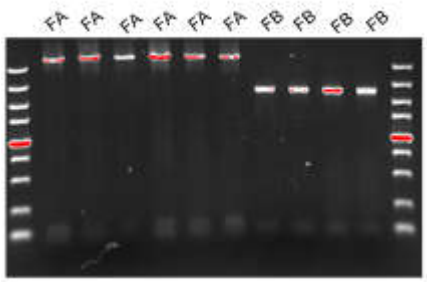


Figure: Electrophoresis result of PCR. The annealing temperature ranges from 55 to 59 ℃.

We chose the optimal annealing temperature as 58.5℃.

Date: 7.28

1. Connect the two fragments using SLIC.

- The system:
  - The system1 for digestion:

	Fragment A	Fragment B
T4 polymerase(0.5U/ul)	2.2ul	1.3ul
Total DNA	12ul	12ul
T4 polymerase buffer	1.6ul	1.6ul

  - The system2 for ligation:

Fragment A	9ul
Fragment B	9ul
Ligase buffer	2ul
Total	20ul
Dilute -fragment A	4.3ul ddH2O+2ul
Dilute-fragment B	8.3ul ddH2O+2ul

- The steps:
  - Incubate 50ul PCR product with 0.5ul DpnI at 37℃ for 30 minutes.
  - Purify the PCR product. (The final volume is 20ul.)
  - Calculate the amount of DNA to be added.
  - Calculate the amount of T4 DNA polymerase (0.5U\ug) to be added.
  - After adding buffer and enzyme, incubate at 37℃ for 30 minutes.
  - Placed on ice quickly.
  - Add 1\10 volume 10mM dCTP to stop the reaction.
  - Store at -20℃.

2. Transformation

- The results:
  - No colony. We speculate that the amount of enzyme is too much, which influences the reaction.

Date: 7.30

1. Connect the two fragments using SLIC.

- The system:
  - The system1:

	Fragment A	Fragment B

Fragment	12ul	12ul
T4 polymerase	2.6ul	1.8ul
Buffer	1.6ul	1.5ul

- The system2:

Fragment A	9ul
Fragment B	9ul
Ligase buffer	2ul
Total	20ul
Dilute -fragment A	5.8ul ddH2O+2ul
Dilute-fragment B	12.9ul ddH2O+2ul

2. Amplify the two fragments on the plasmid by PCR.

- The total volume is increased to 300ul.

3. Measure the conversion efficiency of competent state.

- The results:
  The conversion efficiency of competent trans5a is about 1E+9.

Date: 7.31

1. Amplify the two fragments on the plasmid by PCR.

- The system:

	Fragment A	Fragment B
2x High-Fidelity Master Mix	100ul	50ul
Primer A (10uM)	8ul	4ul
Primer B (10uM)	8ul	4ul
Template DNA (diluted 200 times)	3ul	1.5ul
ddH2O	81ul	40.5ul
Total	200ul	100ul

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

Figure: Electrophoresis result of fragment A and fragment B which are amplified by PCR.

2. Purify the fragments through gel extraction and quantify them.
3. Connect the two fragments using SLIC.

- The system:
  - The system1:

	Fragment A	Fragment B
Fragment	12ul	12ul
T4 polymerase(0.5U/ul)	0.96ul	0.662ul
Buffer	1.55ul	1.48ul

- The system2:

Fragment A	9ul
Fragment B	9ul
Ligase buffer	2ul
Total	20ul
Dilute-fragment B	7.12ul ddH2O+4ul

- The steps are the same as before.
- The results:
  No colony.

Date: 8.1

1. Activating the glycerol bacteria with the dCas9 plasmids (pdCas9).

Date: 8.2

1. Connect the two fragments using SLIC.
- The system and the steps are the same as before.
2. Transformation
3. Plasmid extraction, gel electrophoresis and sequencing.

Date: 8.3

1. Pick the monoclonal colonies of the plate on July 30th.
2. Colony PCR.
3. Plasmid extraction, gel electrophoresis and sequencing.
4. Amplify the two fragments on the plasmid by PCR.

- The system:
  The system is the same as before. However, there is an extra system.

PCR system	50ul
Dpn I	1ul
10x T buffer	5.6ul

- The steps:
  The steps are the same as before. But after PCR, we add Dpn I to digest the template.
  Note: This operation can remove the template DNA and avoid false positive results.
- The results:
  The bands of fragment A are not clear.

Date: 8.4

1. Amplify the fragment A by PCR.
   Note: At the first time, we repeat the experiment. At the second time, we change into new primers and new template. Compared with the first one, the results of the second time indicate that this may be a problem with primers and templates.
2. Gel extraction.

Date: 8.5

1. Amplify the fragment A by PCR.
   Note: We use DpnI in group2-5, while we don't add the enzyme in group1.
- The results:
  Compared with group1, the bands in group2-5 are clearer.

2. Connect the two fragments using SLIC.

- The system:
  - The system1:

	Fragment A	Fragment B
Fragment	12ul	12ul
T4 polymerase	1.0ul	1.2ul
Buffer	1.4ul	1.5ul
	…	…
dCTP	1.4ul	1.5ul

  - The system2:

Fragment A : Fragment B	1:1	1:2
Fragment A	4.5ul	4.5ul
Fragment B	4.5ul	4.5ul
Ligase buffer	2ul	2ul
Dilute-fragment B	5.34ul ddH2O+2ul	1.67ul ddH2O+2ul

Date: 8.6

1. Transformation

- The results:
  No colony.

Date: 8.10

1. Activating the glycerol bacteria with the dCas9 plasmids (pdCas9).
2. Explore the condition to amplify the fragment on the plasmid by PCR.
   Note: we use Primestar as the enzyme which is different with Mclab.

- The system:

5x PS GXL buffer	40ul
dNTP	16ul
Primer1	4ul
Primer2	4ul
Template (2ng/ul)	8ul
Primestar GXL polymerase	8ul
ddH2O	120ul
Total	200ul

- The steps:

98℃	30s
98℃	10s x30 cycle
--52℃~58℃	15s
68℃	5min
4℃	Hold

- The results:

  We choose 55℃ as the optimal annealing temperature.

3. Amplify the fragment on the plasmid by PCR.
- The steps are the same as the optimal steps.

Date: 8.11

1. Plasmid extraction and nucleic acid quantification.
- The results:

dCas9 plasmid	Concentration
1	23
2	20
3	26
4	27
5	43
6	28

2. Enzyme digestion:
- The principle:
  We use new way to construct the plasmid. The backbone is constructed by enzyme digestion of original plasmid. The fragment is constructed by PCR. The designed primers can add restriction enzyme cutting site. Through enzyme digestion, the fragment can expose the same sticky ends.
- The system:

	Fragment A (with dCas9)	Fragment B
DNA	24ul	14ul
10x Quickcut buffer	3ul	2ul
Q.EcoR I	1ul	1ul
Q.Xba I	1ul	1ul
ddH2O	1ul	2ul
Total	30ul	20ul

- The steps:

	Fragment A	Fragment B
37℃	45 minutes	15 minutes
-20℃	-	Hold

- The results: ~酶切结果

3. Gel extraction and nucleic acid quantification.
4. Ligation:

- The system:

	Group1	Group2
10x ligase buffer	1.5ul	2ul
plasmid	3.5ul	7ul
insert	5.2ul	10.2ul
T4 ligase	1ul	1ul
ddH2O	3.8ul	0.8ul
Total	15ul	20ul

- The steps:
  - Mix the reaction system.
  - The mix are placed at 16℃ for 8h and then at 4℃ overnight.

5. Activating the glycerol bacteria with the dCas9 plasmids (pdCas9).

Date: 8.12

1. Enzyme digestion, gel extraction and ligation.
2. Transformation.
3. Plasmid extraction and nucleic acid quantification.

Date: 8.13

1. Pick the monoclonal colonies. Do colony PCR. And add liquid culture medium to amplify the bacteria.

Date: 8.14

1. Plasmid extraction and sequencing.

- The results:
  The concentration of the plasmid is too low to measure.

2. Pick new monoclonal colonies and colony PCR.

- The results:
  The bacteria don't grow.

3. Prepare solid and liquid culture medium.

Date: 8.15

1. Enzyme digestion, gel extraction and ligation.
   Note: At this time, we change the system of ligation.

- The system:

10x T4 ligase buffer	1.5ul
Backbone(20ng)	10ul
Fragment(40ng)	2ul
T4 ligase	1ul
ddH2O	0.5ul
Total	15ul

2. Transformation.

Date: 8.17

1. Pick the monoclonal colonies. And add liquid culture medium to amplify the bacteria.

- The results:
  The bacteria don't grow, which indicates that tetracycline doesn't work.

2. PCR the recombinant plasmid

- The principle:
  If the electrophoresis result of the recombinant plasmid is the same as the original plasmid, the two fragments connect correctly. If not, they don't.
- The results:
  There is no the same results. Therefore, they connect incorrectly.

3. Amplify the fragment on the plasmid by PCR.
4. Gel extraction.
5. Enzyme digestion, gel extraction and ligation.

- The system:

	Backbone	Fragment
DNA	14ul	5ul
10x Q.buffer	2ul	1.5ul
Q.EcoR I	1ul	1ul
Q.Xba I	1ul	1ul
ddH2O	2ul	6.5ul
Total	20ul	15ul

- The steps:

	Backbone	Fragment

37℃	45min+20min	25min
4℃	Hold	Hold

Date: 8.18

1. Transformation.

- The results:
  No colony.

Date: 8.19

1. Enzyme digestion, gel extraction and ligation.

Date: 8.20

1. Transformation.
2. Enzyme digestion, gel extraction and ligation

- The system:

	Backbone	Fragment
DNA	10ul	3ul
10x Q.buffer	1.5ul	1ul
Q.EcoR I	1ul	1ul
Q.Xba I	1ul	1ul
ddH2O	1.5ul	4ul
Total	15ul	10ul

- The steps:

	Backbone	Fragment
37℃	1.5h	1.5h
4℃	Hold	Hold

  Note: We digest for 1.5h which is longer than before. And we use NEB ligase to connect the fragment.

Date: 8.21

1. Transformation.

2. Explore the condition to amplify the fragment by PCR using new primers.

- The system:

2x Mix	100ul
Primer1	6ul
Primer2	6ul
Template	4ul
ddH2O	84ul
Total	200ul

- The results:

  The optimal annealing temperature is 54℃。

3. Amplify the fragment by PCR under the optimal condition.

Date: 8.22

1. Enzyme digestion of the backbone and the amplified fragment.

- The principle:
  According to the design of the new primer, we use EcoR I and Xho I as the enzyme.
- The steps:

  We incubate at 37℃ for 1h.

2. Gel extraction and nucleic acid quantification.
3. Ligation:

- The steps:
  We connect the fragments in group1 for 8h, while we connect the fragments in group2 for 14h.

Date: 8.23

1. Transformation.

- The results:
  NC group doesn't have colony. PC group and experimental groups have colonies.

Date: 8.24

1. Pick the monoclonal colonies. And do colony PCR.
2. Add medium to the colonies with positive results.