Molecular Cloning Protocol

[Golden Gate Cloning]

The enzymes and other reagents are bought from NEB.

20 μL system

2 μL 10X T4 ligase buffer

1 μL T4 ligase

1 μL BsaI-HF

DNA solution

add ddH2O to 20 μL

The procedures of Golden Gate Cloning

Step1. Prepare 20 μL system ut supra.As for the DNA solution, according to our experience, 40 fmol vector and 1 μL sgRNA fragment prepared works extremely finely.NEB also has its online tool to calculate the DNA amount:
https://www.neb.com/external-links/neb-golden-gate-assembly-tool

Step2. Run the following program in the PCR system:

Program

Process	Temperature	time
1	37°C	5min
2	16°C	5min
Go to 2	cycle number	16X
3	37°C	15mi
4	50°C	5min
5	80°C	5min
6	16°C	∞

Step3. Transfer the recombination DNA into competent E. coli cells.

Step4. Add 100 μL LB into each tube and put in the shaker at 37 ℃ 220 rpm to recover 1 h.

Step5. Put on the surface of LB medic and culture at 37 ℃ overnight (about 14-16 h).

[Gradient Annealing]

20 μL system

FW single strand DNA 10 μL

RV single strand DNA 10 μL

Program

Process	Temperature	time
1	98°C	5min

2	95°C	1.5min
3	92°C	1.5min
4	90°C	1min
5	88°C	1min
6	85°C	1min
7	83°C	1min
8	78°C	1min
9	75°C	1min
10	72.5°C	1min
11	70°C	1min
12	68°C	1min
13	65°C	1min
14	62.5°C	1min
15	60°C	1min
16	57.5°C	1min
17	52.5°C	1min
18	50°C	1min
19	45°C	1min
20	40°C	1min
21	35°C	1min
22	30°C	1min
23	25°C	1min
24	20°C	1min
25	16°C	∞

Dilute to 1/10 before use.

[Gibson Assembly]

We use the Gibson assembly method based on Miller Lab's protocol. The enzymes and other reagents are bought from NEB. We use SnapGene to assist the primer designing.

The preparation of ISO buffer

(Miller Lab protocol, http://miller-lab.net/MillerLab/protocols/molecular-biology- and-cloning/gibson-assembly/ and-cloning/gibson-assembly/)

3 ml of 1 M Tris-HCl pH 7.5
150 µL of 2 M $MgCl_2$
60 µL of 100 mM dGTP
60 µL of 100 mM dATP

60 μL of 100 mM dTTP
60 μL of 100 mM dCTP
300 μL of 1 M DTT
1.5 g PEG-8000
300 μL of 100 mM NAD
Add water to 6 mL
Aliquot 100 μL and store at -20 °C
The preparation of Gibson assembly mix
320 μL 5X ISO buffer
0.64 μL of 10 U/μL T5 exonuclease 20 μL of 2 U/μL Phusion polymerase 160 μL of 40 U/μL Taq ligase
Add water to 1.2 mL
Aliquot 15 μL and store at -20 °C.

The procedures of Gibson assembly
Step1. Take 5 μL DNA solution into 15 μL Gibson assembly mix. The molecule ratio of insert DNA segment and vector backbone should be about 3:1 to get prefer effect.
Step2. Put the reaction tube in metal bath at 50 ℃ for 1 h.
Step3. Transfer the recombination DNA into competent *E. coli* cells.
Step4. Add 100 μL LB into each tube and put in the shaker at 37 ℃ 220 rpm to recover 1 h.
Step5. Put on the surface of LB medic and culture at 37 ℃ overnight (about 14- 16 h).

[Restriction Recombination]
We use restriction endonuclease and T4 DNA ligase to do the restriction recombination. These reagents are bought from NEB.

Step1. Use 1 μL of each kind of restriction endonuclease to cut DNA segment in 50 μL system each tube reaction at 37 ℃ metal bath. To cut 500 ng DNA at least use 1 U enzyme pre hour. Keep at 65 ℃ (some enzymes need 80 ℃) for 20 min to inactivate the enzyme.
Step2. Recycle the target DNA segment after cutting through gel electrophoresis and extraction.
Step3. Mixed insert DNA segment and vector backbone as molecule ratio right to be about 3:1 with a total volume of 10 μL system. Use 1 μL T4 DNA ligase in each tube reaction and keep at 16 ℃ overnight (about 16 h).

Step4. Transfer the recombination DNA into competent E. coli cells.
Step5. Add 100 μL LB into each tube and put in the shaker at 37 ℃ 220 rpm to recover 1 h.
Step6. Put on the surface of LB medic and culture at 37 ℃ overnight (about 14-16 h).