1. Obtain *hmaS* mutant fragment by PCR

   Adjust the concentration of Mn^{2+} and Mg^{2+} to determine the appropriate mutation rate

PCR system (50 μL)	
Scpa1-F	2 μL
Hmas-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2 x Mix	25 μL
Mn^{2+} (1 mM)	2 μL
Mg^{2+} (25 mM)	2.5 μL
dNTP	1 μL
DDW	14.5 μL

PCR system (50 μL)	
Scpa1-F	2 μL
Hmas-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2 x Mix	25 μL
Mn^{2+} (1 mM)	0.5 μL
Mg^{2+} (25 mM)	2.5 μL
dNTP	1 μL
DDW	16 μL

PCR system (50 μL)	
Scpa1-F	2 μL
Hmas-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2 x Mix	25 μL
Mn^{2+} (1 mM)	1 μL
Mg^{2+} (25 mM)	2.5 μL
dNTP	1 μL
DDW	15.5 μL

PCR system (50μL)	
Scpa1-F	2 μL
Hmas-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2xMix	25 μL
Mn^{2+} (1 mM)	1.5 μL
Mg^{2+} (25 mM)	2.5 μL
dNTP	1 μL
DDW	15 μL

The target fragment is located on the Pccd-K-scpa1 plasmid, and the amplified target fragment is obtained by PCR.

The PCR products are detected by agarose gel electrophoresis, and the correct target fragment is 1248bp. We obtained the correct target fragment, and the sample is purified.

1. Obtain Bio177 vector fragment by PCR

The target fragment is located on the pYB1a-Bio177-Str plasmid, and the amplified target fragment is obtained by PCR. The PCR system and procedure are showed below.

PCR system (50 μL)	
Bio177-F	2 μL
Bio177-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2 x Mix	25 μL
DDW	20 μL

The PCR products are detected by agarose gel electrophoresis, and the correct target fragment is 5089bp. We obtained the correct target fragment, and the sample is purified.

2. Golden gate assembly

The *hmaS* mutant fragments and Bio177 vector fragments are connected by Golden gate assembly method, and the system is as follows.

Golden gate system (50 μL)	
hmaS mut	1 μL
Bio177	1 μL
T4 Buffer	1 μL
rCutsmart	1 μL
T4 ligase	0.2 μL
BsaI	0.5 μL
DDW	5.3 μL

Golden gate	
37 ℃	60 min
55 ℃	15 min
80 ℃	15 min

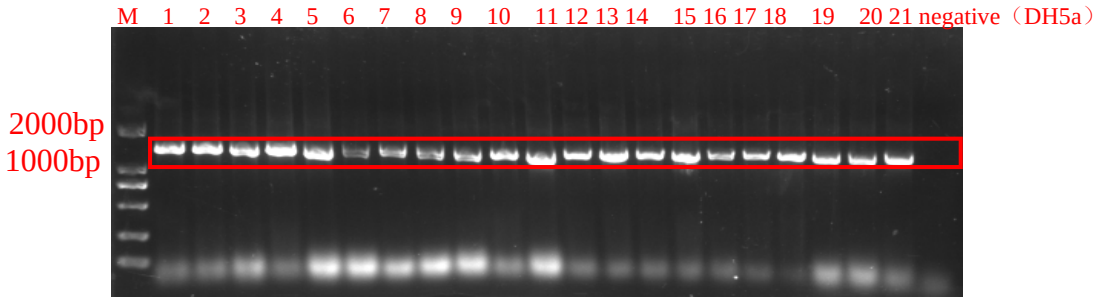
3. Colony PCR

After cultured at 37°C for 12 hours, 30 colonies were selected on the plate. The colony PCR system and procedure were as follows.

PCR system (50 μL)	
Bio177-F	2 μL

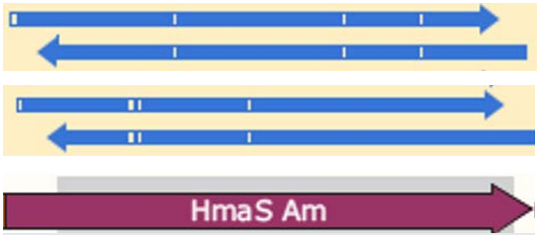
Bio177-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2 x Mix	25 μL
DDW	20 μL

The PCR products were detected by agarose gel electrophoresis, and the results were as follows.



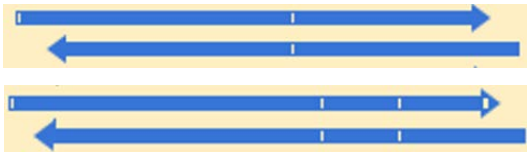
4. Sequencing for ensured appropriate mutation rate

Group 1: The final concentration of Mn^{2+} is 0.04 mM



Group 2: The final concentration of Mn^{2+} is 0.03 mM

Group 3: The final concentration of Mn^{2+} is 0.02 mM



Group 4: The final concentration of Mn^{2+} is 0.01 mM