Molecular Biotechnology

We used different molecular biological methods in our project. All used methods are listed below.

1. Genome preparation

In our project, we use the *Magnetospirillum gryphiswaldense MSR-1* genome as template to amplify our target gene. Genome preparation is a method for isolating Genome from bacterial cell cultures. In this work the TIANamp Bacteria DNA Kit (Tiangen) was used. The Genome preparation was performed following the manufacturer's manual.

1. PCR

In addition to the common PCR for amplification of certain DNA fragments, several different types of PCR were used throughout our project. The purpose, procedure and generic use are listed in the table below.

Name	Purpose	Procedure	Notes
Common PCR	amplification of certain DNA fragments	Primers binding in the side of the target genes.	general reaction setup and procedure shown in tables below
colony/check PCR	Check on insert length/ correct integration of insert	primers binding in the middle of the insert or one in insert and one in vector are used	general reaction setup and procedure shown in tables below
SOE PCR	side directed mutagenesis	primers containing the desired base pair exchange/deletion/insertion are designed and used	**S**plicing by **O**verlapping **E**xtension PCR
gradient PCR	Finding optimal conditions for our primers to bind and the PCRs in general	Several PCRs batches are run within the same thermocycler, differing in annealing temperature	Commonly used in preliminary experiments or appear nonspecific binding.

General reaction setup of a common PCR

component	volume	
Q5 Hot Start High-Fidelity 2X Master Mix （Purchased From NEB）	25µl	12.5µl
10µM Forward primer	2.5µl	1.25µl
10µM Reverse primer	2.5µl	1.25µl
Template	1µl（<1000ng）	0.5µl
ddH_2O	19µl	9.5µl

General reaction procedure of a Common PCR

pre-denaturation	98°C	30s	
denature	98°C	10s	35 cycles
anneal	Tm	30s	
elongate	72°C	20-30s per kb	
elongate	72°C	5min	
store	12°C	∞	

General reaction setup of a colony/check PCR

component	volume
2xTaq PCR Master Mix（Purchased From Tiangen）	10 μl
bacterial suspension	2μl
10μM Forward primer	1 μl
10μM Reverse primer	1 μl
ddH_2O	6μl

Pick colony with tooth picks and suspend in 10μl ddH_2O as bacterial suspension

General reaction procedure of a colony/check PCR

pre-denaturation	94°C	3min	
denature	94°C	30s	30 cycles
anneal	58°C	30s	
elongate	72°C	1min per kb	
elongate	72°C	3min	
store	12°C	∞	

2. DNA-Purification

Some molecular biological methods require a purification of DNA after amplification or modification. In this work, the Universal DNA Purification Kit (Tiangen) was used. In the presence of chaotropic salts the nucleic acids are bound the glass fiber fleece in the Filter Tube while other substances are removed by the washing steps. Afterwards purified DNA fragments are be eluted with deionized water. Unless stated otherwise the DNA purification was performed following the manufacturer's manual.

3. Cloning vector Ligation

Add component in the microcentrifuge tube as follow:

PCR products 0.5-4ul(available, don't beyond 4ul)

pEASY-Blunt Simple Cloning Vector(TransGene Biotech) 1ul

Mix it gently, reaction at 20°C -37°C for 5min, and the put the microcentrifuge tube on ice.

The optimum reaction time:

(1) Fragment length: 0.1-1kb 5-10min;

(2) Fragment length:1-2kb 10-15min;

(3) Fragment length:2-3kb 15-20min;

4. Plasmid Preparation

Plasmid preparation is a method for isolating plasmids from bacterial cell cultures. In this work the *EasyPure* Plasmid MiniPrep Kit (Trans Gene Biotech) was used. After the cells are lysed, the lysate is applied to a mini column binding plasmid DNA to a silica membrane in the presence of chaotropic salts. Following a washing step, the DNA is eluted with deionized water. Unless stated otherwise the plasmid preparation was performed following the manufacturer's manual.

5. Double enzymes restriction digest

Restriction endonucleases are used to cut double stranded DNA molecules at specific, usually palindromic base sequences. Reactions setup as blow:

1. Prepare the reaction mixture at room temperature in the order indicated:

Component	Volume	
	Plasmid DNA	Unpurified PCR product
Water, nuclease-free	35 μl	17 μl

10X FastDigest® buffer or 10X FastDigest® Green buffer	5 µl	2 µl
DNA	5 µl (up to 2µg)	10 µl (~0.2 µg)
FastDigest® enzyme1（NEB）	2.5 µl	1 µl
FastDigest® enzyme2（NEB）	2.5 µl	1 µl
Total volume	50 µl	30 µl

2. Mix gently and spin down.
3. Incubate at 37°C in a heat block or water thermostat for 30 min.
4. Inactivate the enzyme (optional).

2. Ligation

Ligation in the context of molecular biology is the enzymatic joining of previously restricted nucleic acid fragments by synthesis of new bonds with simultaneous breakdown of ATP.

1. Set up the following reaction in a microcentrifuge tube on ice.

(T4 DNA Ligase was added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.

COMPONENT	20 µl REACTION
10X T4 DNA Ligase Buffer*	2 µl
Vector DNA :Insert DNA	1:3
Nuclease-free water	to 20 µl
T4 DNA Ligase(NEB)	1 µl

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. incubate at 16°C overnight.
4. Heat inactivate at 65°C for 10 minutes（optional）.
5. Chill on ice and transform 5-10 µl of the reaction into 100 µl competent cells.

3. Gibson Assembly

Gibson assembly is a novel method for the easy assembly of multiple linear DNA fragments. Regardless of fragment length or end compatibility, multiple overlapping DNA fragments can be joined in a single isothermal reaction. With the activities of three different enzymes, the product of a Gibson Assembly is a fully ligated double-stranded DNA molecule. In this work the *pEASY*-Uni Seamless Cloning and Assembly Kit (Trans Gene Biotech) was used.

1. Set up the following reaction in a microcentrifuge tube on ice.

2XAssmbly Mix	5µl
Linearized vector	X µl
Inserts	Y µl
ddH_2O	To 10µl

The molar ratio of X: Y is 1:2.

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. Incubate at 50°C for 15min.
4. Chill on ice for several seconds and transform 5-10 µl of the reaction into 100 µl competent cells.

4. Transformation

The induction of competence of bacterial cells as well as the uptake of exogenous genetic material by these cells from their surroundings was done by using heat-shock transformation

Heat-Shock

1. Thaw cells on ice.
2. Add 1 µl of plasmid DNA.
3. Incubate on ice for 30 min.
4. Heat shock at 42°C for 60 s.
5. Incubate on ice for 3 min.
6. Add 350 µl of SOC media.
7. Incubate at 37°C for 1 h.
8. Plate 100µl on plates supplemented with the appropriate antibiotic.