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Molecular Cloning

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ABSTRACT

SZPT-CHINA Experimental methods of molecular cloning

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MATERIALS TEXT

MATERIALS

Q5 High-Fidelity 2X Master Mix - 500 rxns **New England Biolabs Catalog #M0492L**

Nuclease-free Water **New England Biolabs Catalog #E6317**

NEBNext Second Strand Synthesis Enzyme Mix **New England Biolabs Catalog #E7425**

Nuclease-free Water **New England Biolabs Catalog #E7764**

T4 DNA Ligase Buffer (10X) **Thermo Fisher Catalog #B69**

2×PCR Master Mix **Solarbio**

Vector linearization and target fragment preparation

1 Q5 DNA polymerase PCR system(**50 µL**)

25 µL Q5 High-Fidelity 2X Master Mix

2 µL 10µL Forward Primer

2 µL 10 µM Reverse Primer

1 µL Template DNA

20 µL Nuclease-Free Water

After mixing gently, centrifuge to collect all the liquid to the bottom.

PCR Program

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
Denaturation	98°C	5 seconds
Annealing	*50–72°C	10 seconds
Extension	72°C	15 seconds/kb
25–35 Cycles		
Final Extension	72°C	2 minutes
Hold	4–10°C	

Enzyme digestion Connection

2 Enzyme digestion Connection

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2.1 Enzyme digestion and connection system(**10 μL**)

3 μL Vector

1 μL Insert

1 μL T4 DNA Ligase Buffer (10X)

0.5 μL NEB Golden Gate Enzyme Mix (BsaI-HFv2)

4.5 μL Nuclease-free H2O

2.2 Connection system

INSERT NUMBER	SUGGESTED ASSEMBLY PROTOCOL
For 1 Insert	37°C, 5 min (cloning) or 37°C, 1 hr (library preparation) ® 60°C, 5 min
For 2–10 Inserts	(37°C, 1 min ® 16°C, 1 min) x 30 ® 60°C, 5 min
For 11–20+ Inserts	(37°C, 5 min ® 16°C, 5 min) x 30 ® 60°C, 5 min

Transformation

3 Transformation

3.1 Take 50 μL of competent cells in a sterile Eppendorf tube.

3.2 Add 2~5 μL of the plasmid to be transformed into each tube, gently mix the plasmid and competent cells with a pipette tip, and ice bath for 25 minutes.

3.3 Heat shock the ice-bath mixture in a 42°C water bath for 45s, do not shake the centrifuge tube.

3.4 Take out the ice bath and cool for 2 minutes.

3.5 Add 800 μL of non-resistant LB medium preheated to 37°C to each tube, shake gently at 37°C, 150r/min for 1h.

3.6 Centrifugally concentrate to 100μL, spread it on an LB plate containing antibiotics, and incubate inverted at 37°C for 12-16h.

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3.7 Make a negative control at the same time (use distilled water instead of plasmid).

Colony PCR screening of positive clones

4 Colony PCR screening of positive clones

4.1 Add 25ul of sterile water to the 96-well plate, pick about 15-20 colonies from the successfully transformed DH5α bacterial plate with a pipette tip, and mix them evenly. (Make a corresponding mark on the picking plate).

4.2 Put the mixed bacteria solution into 10ul to 200ul PCR tube and mark it.

4.3 Take 0.1ul of bacterial solution in a 96-well plate and add it to the antibiotic-containing plate with a pipette tip and mark it.

4.4 After mixing, the bacteria solution is used for PCR, the system is as follows(**20 μL**)

- **5 μL 2×PCR Mix**
- **0.5 μL 10μM Forward Primer**
- **0.5 μL 10 μM Reverse Primer**
- **2 μL Bacteria solution**
- **2 μL dd H2O**

The procedure is as follows

STEP	TEMP	TIME
Initial Denaturation	94°C	30 seconds
Denaturation	94°C	3min
Annealing	*55-60°C	30seconds
Extension	72°C	1min/kb
25–35 Cycles		
Final Extension	72°C	2 minutes
Hold	4–10°C	

4.5 Verification result of 1% agarose gel electrophoresis.

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