2024.6.8-2024.7.1

1. **Preparation of Nissle1917 (DE3) Competent Cells**

(1) Bacterial culture of Nissle1917: Inoculate Nissle1917 into 200 mL of LB liquid culture meduim and incubate in a 37℃ shaker.

(2) Collect the bacterial cells using four 50 mL round-bottom centrifuge tubes (at 25℃, 4,000 rpm for 10 minutes). After centrifugation, carefully discard the supernatant in a super-clean bench.

(3) Add approximately 5 mL of sterile water to each tube to resuspend the bacterial cells. Pool the bacterial cells from the four tubes into one centrifuge tube, and add sterile water to make the total volume up to 40 mL. Resuspend and ish the bacterial cells. Prepare another centrifuge tube of the same weight and centrifuge them together. After centrifugation (at 25℃, 4,000 rpm for 10 minutes), carefully discard the supernatant in the super-clean bench.

(4) Resuspend and ish the bacterial cells with 40 mL of 10% glycerol. After centrifugation (at 25℃, 4,000 rpm for 10 minutes), carefully discard the supernatant in the super-clean bench.

(5) Resuspend and ish the bacterial cells again with 40 mL of 10% glycerol. After centrifugation (at 25℃, 4,000 rpm for 10 minutes), carefully discard the supernatant in the super-clean bench.

(6) Resuspend the bacterial cells with 200 µL of 10% glycerol. Aliquot 50 µL of the bacterial suspension into 1.5 mL EP tubes. Label each tube as Nissle1917. Flash-freeze the tubes in liquid nitrogen and store them in a -80℃ freezer.

2. Polymerase chain reaction

(1) Primers:

T5-sfGFP-32a (cSAT) Arm-F T5-sfGFP-32a (cSAT) Arm-R

DNA: Fragment sfGFP15 (containing 32a-cSAT) Size: 756 bp

Result: Perform agarose gel electrophoresis. After verifying the correctness by running the test gel, run the recovery gel. Cut the gel block containing the target band and conduct gel recovery. The obtained plasmid fragment concentration is 32 ng/µL.

(2) Primers:

pET32a-cSAT-Retroextension-F pET32a-Retroextension-R (2)

DNA: pET32a (linearized empty vector) Size: 5714 bp

Result: The concentration of the recovered plasmid fragment is 8.5 ng/µL.

(3) Primers:

V-pTZ28a-F new 3Csite-R

DNA: pTZ28a-sfGFP-3C (linear) Size: 6398 bp

Result: The recovered plasmid concentration is 7.7 ng/μL.

(4) Primers:

4GLP-1/LV-F 4GLP-1/LV-R

DNA: Fragment 4GLP-1-5LV (DDDDK) Size: 672 bp

Result: The recovered plasmid concentration is 39 ng/μL.

(5) Primers:

pET32a-cSAT-Retroextension-F pET32a-Retroextension-R

DNA: pET32a (linear, non-self-ligated) Size: 5699 bp

Result: The recovered plasmid concentration is 11.9 ng/μL.

(6) Primers:

GLP-1-F GLP-R

DNA: DH5α pET32a empty vector circularized and self-ligated Size: 3978 bp

Result: There is a band and the band size is correct.

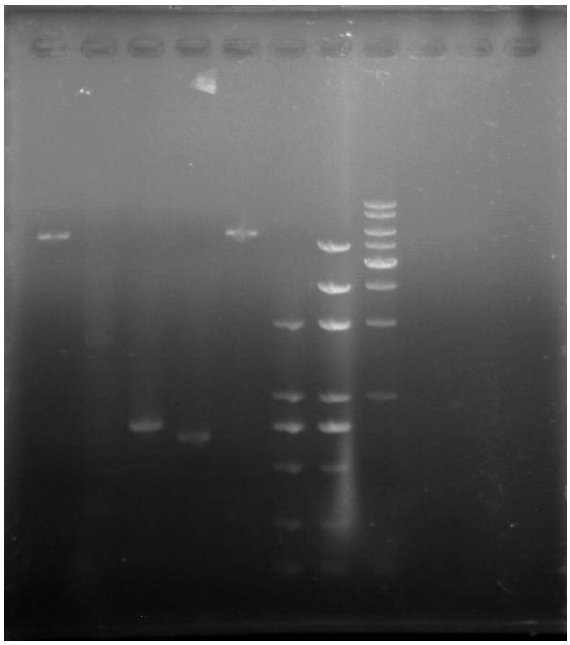

Figure 4

3. T5 Ligation

(1) For the sfGFP fragment and the pET32a-His6, based on a molar ratio of fragment

to vector of ≥ 3:1, 3.0944 μL of the fragment and 0.08 μL of the vector are required. Water is added to make the total volume up to 4 μL. Subsequently, 0.5 μL of buffer and diluted T5 enzyme each are added. Under the T5 ligation reaction conditions, the fragments are ligated together by the T5 enzyme to form the plasmid.

pET32a-cSAT-sfGFP: sfGFP + pET32a-His6 —— pET32a-cSAT-sfGFP

(2) For the cSAT-GLP-1 fragment and the pTZ28a-His6-sfGFP, based on a molar ratio of fragment to vector of ≥ 3:1, 2.37 μL of the fragment and 0.15 μL of the vector are required. Water is added to make the total volume up to 4 μL. Subsequently, 0.5 μL of buffer and diluted T5 enzyme each are added. Under the T5 ligation reaction conditions, the fragments are ligated together by the T5 enzyme to form the plasmid pTZ28a-sfGFP-cSAT-GLP-1:

cSAT-GLP-1+pTZ28a-sfGFP——pTZ28a-sfGFP-cSAT-GLP-1

(3) For the pET32a-His6 amplified by reverse PCR, 3 μL of the amplified product is added, and 1 μL of water is added to make the total volume up to 4 μL. Then, 0.5 μL of buffer and diluted T5 enzyme each are added. Under the T5 ligation reaction conditions, the vector is self-ligated by the T5 enzyme to form the pET32a-His6.

After the T5 ligation step is completed, it is placed in a 37℃ shaker for 45 minutes to 1 hour. Three plates containing the corresponding antibiotic medium are prepared. On the plates, labels are made indicating the strain, plasmid, date, and the initials of the person's name, such as DH5α pET32a-cSAT-sfGFP 6.30 CLY. Then, in a super-clean bench, the samples are spread onto the culture plates containing the corresponding antibiotic using the spreading plate method and placed in a 37℃ incubator upside down for overnight culture.

4. Monoclonal Colony PCR (T5-Spread Plates)

(1) Primers:

sfGFP15-F T5-cSAT-GLP-28a Arm-R

DNA: DH5a pTZ28a-sfGFP-cSAT-GLP-1 Size: 1446 bp

Result: The size of the agarose gel electrophoresis band is correct when compared to the marker.

(2) Primers:

GLP-1-F GLP-R

DNA: DH5a pET32a-His6 Size: 3978 bp

Result: There is a band in the agarose gel electrophoresis and the size is correct.

5. Polymerase chain reaction

(1) Primers:

T5-4GLP-1/LV-F-32a Arm T5-4GLP-1/LV-R-32a Arm

DNA: 4GLP-1-5LV Size: 723 bp

Result: The concentration of the recovered plasmid fragment is 45.95 ng/μL.

(2) Primers:

T5-4GLP-1/LV-F-28a Arm T5-4GLP-1/LV-R-28a Arm

DNA: 4GLP-1-5LV Size: 709 bp

Result: The concentration of the recovered plasmid fragment is 37.50 ng/μL.

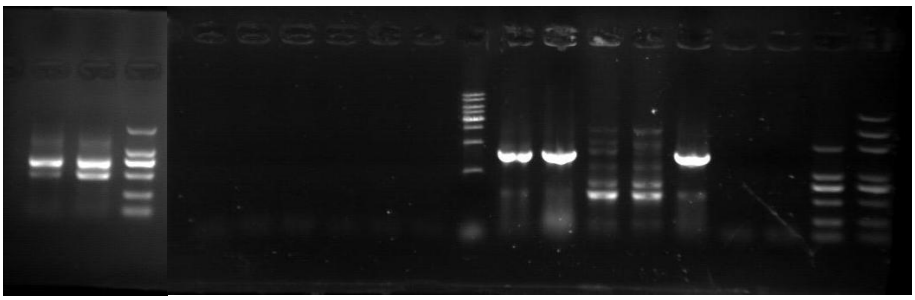

Figure 5

6. pTZ28a-1913-GL-①: The glycerol stock of the bacteria is purified by streaking on LK medium plates using the plate streaking method and then placed in a 37℃ constant temperature incubator for culturing.

7. T5 Enzyme Ligation

(1) For the second group of plasmid fragments recovered from PCR, which contained 4GLP-1-5LV-32a Arm and the pET32a linear non-self-ligated vector, based on a molar ratio of fragment to vector of ≥ 3:1, it is calculated that 3.01 μL of the fragment and 0.4 μL of the vector are required. Water is added to make the total volume 4 μL. Then, 0.5 μL each of buffer and diluted T5 enzyme are added. Through the T5 enzyme ligation reaction, a plasmid is formed:

4GLP-1-5LV-32a Arm + pET32a linear non-self-ligated —— pET32a-4GLP-1-5LV

It is spread on LA medium plates in a clean workbench and placed in a 37℃ constant temperature incubator for overnight culture.

(2) For the third group of plasmid fragments recovered from PCR, which contained 4GLP-1-5LV -28a Arm and the pTZ28a-sfGFP vector, based on a molar ratio of fragment to vector of ≥ 3:1, it is calculated that 2.23 μL of the fragment and 0.15 μL of the vector are required. Water is added to make the total volume 4 μL. Then, 0.5 μL each of buffer and diluted T5 enzyme are added. Through the T5 enzyme ligation reaction, a plasmid is formed:

4GLP-1-5LV-28a Arm + pTZ28a-sfGFP —— pTZ28a-sfGFP-4GLP-1-5LV

It is spread on LK medium plates in a super-clean bench and placed in a 37℃ constant temperature incubator for overnight culture.

(3) For the pTZ28a circularized self-ligated vector recovered from PCR, 3 μL of the vector is added and water is added to make the total volume 4 μL. Then, 0.5 μL each of buffer and diluted T5 enzyme are added. Through the T5 enzyme ligation reaction, a plasmid is formed and transformed into trans110 competent cells:

pTZ28a circularized self-ligation —— pTZ28a-His6-sfGFP

It is spread on LK medium plates in a super-clean bench and placed in a 37℃ constant temperature incubator for overnight culture.

8. 10 μL of glycerol bacteria of pTZ28a-1913-GLP-1 is aspirated and added to 10 mL of LK liquid culture meduim , and then placed in a 37℃ shaker for overnight culture.

9. Bacterial culture

The water-soluble bacteria with correct band sizes in the agarose gel electrophoresis of the monoclonal colony PCR are labeled. 9 μL of each is respectively aspirated and added to 10 ml of liquid culture meduim with corresponding resistance, and placed in a 37℃ shaker for overnight culture. The DH5α pTZ28a-sfGFP-cSAT-GLP-1 strain and the DH5α pET32a -His6 circular self-ligated strain are obtained.