2024.8.8-2024.9.20

1. **Aspirate 9 μL of BL21 pET32a-GLP-1 30 μL bacterial solution into a centrifuge tube containing 7 mL of LA liquid culture meduim. Prepare four groups in parallel, and placed into a 37℃ shaker for overnight culture.**

2. **Measure the OD600nm of the bacterial liquid, and turn to small-scale system （5 mL of LA liquid culture meduim), put into a 37℃ shaker to culture. When the OD600nm ranges from 0.6 to 0.8, cool the system to 18℃, and the system followed by the addition of IPTG for inducing expression for 24 hours. Continue culturing, measure the OD600nm value, transfer to a larger system (containing 150 mL of LA liquid culture medium), and place it in a 37℃ shaker for culturing. When the OD600nm value ranges from 0.6 to 0.8, cool the system to 18℃, followed by the addition of 200 μL of IPTG for inducing expression for 24 hours.**

3. **Culture competent cells of BL21(DE3), obsorb 30 μL bacteria liquid to 30 mL of LA liquid culture meduim, put into 37℃ shaker for overnight culture.**

4. **Bacteria collection**

For the small-scale system, the bacteria are collected using 50 mL centrifuge tubes. The centrifuge is operated at 4000 rpm for 10 minutes. Before centrifugation, balance the tubes.

5. **Resuspend**

The strains are collected after centrifugation, resuspend them with 10 mL buffer. Buffer formula: 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 8.5.

6. **Break bacteria**

The resuspended bacterial liquid is broken by an ultrasonic cell grinder, and each tube is broken three times.

7. **Centrifugation**

For the crushed bacterial samples, 2 mL of each sample is aspirated into 2.0 mL EP tubes, two tubes for each sample. The samples are centrifuged at 12,000 rpm for 30 minutes, and then placed in a refrigerator at 4℃.

8. **Prepare protein gel** Prepare one plate of 10-well protein gel.

9. **Sample preparation** The sample is boiled in a metal bath at 100℃ above 20 minutes.

10. Load the sample

BL21(DE3) pET32a-cSAT-GLP-1:

supernatant① supernatant② supernatant③ supernatant④

precipitation① precipitation② precipitation③ precipitation④

marker marker

11. Run the protein gel on SDS-PAGE for about 50 minutes, then stain it with the staining solution.

12. Decolorization and photoresist

After the protein gel is decolored with water and decolorizing solution, the gel is illuminutesated to observe the bands.

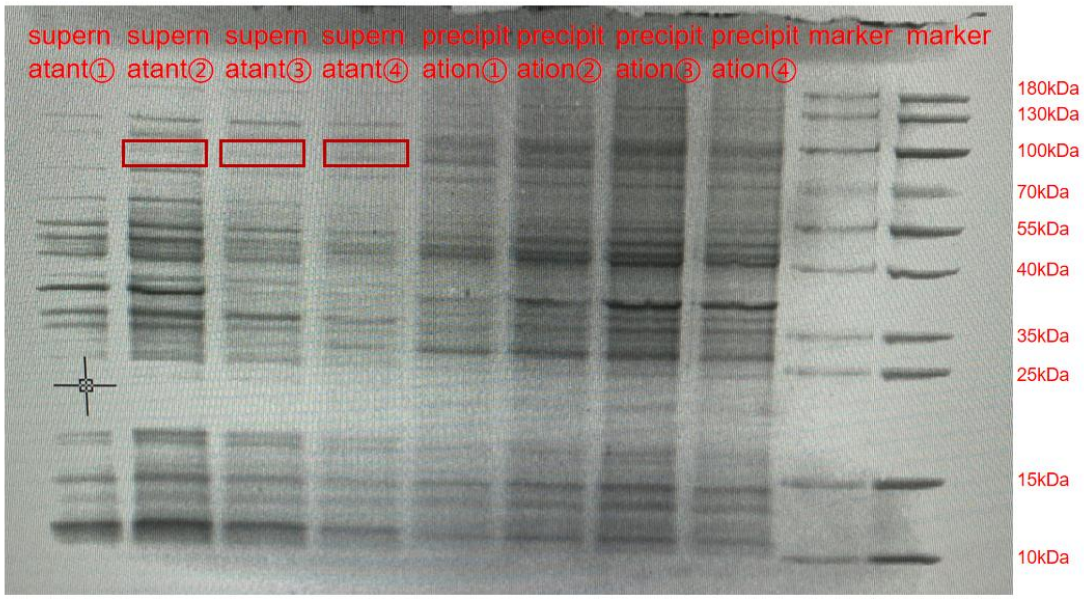

Figure 9 BL21 pET32a-cSAT-GLP-1 small-scale system

13. Collect bacteria

In a large-scale system, the bacteria are collected using 50 mL centrifuge tubes and centrifuged at 4000 rpm for 10 minutes. Before centrifugation, balance the tubes.

14. Resuspend

The strains collected after centrifugation are resuscitated with 15 mL buffer. Buffer formula: 20 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.5.

15. Break bacteria

The resuspended bacterial liquid is broken by an ultrasonic cell grinder, and each tube is broken three times.

16. Centrifugation

For the crushed bacterial samples, 2 mL of each sample is aspirated into 2.0 mL EP tubes, two tubes for each sample. The samples are centrifuged at 12,000 rpm for 30 minutes, and then placed in a refrigerator at 4℃.

17. **Incubated, purified, and eluted protein with the concentration gradients of 10 mM,30 mM, 50 mM, 80 mM, 300 mM imidazole.**

18. **Prepare protein gel** Prepare one plate of 10-well protein gel.

19. **Sample preparation** The sample is boiled in a metal bath at 100℃ above 20 minutes.

20. **Load the sample**

BL21(DE3) pET32a-cSAT-GLP-1:

flow-through flow-through 10 mM imidazole 10 mM imidazole

30 mM imidazole 30 mM imidazole 50 mM imidazole 50 mM imidazole marker

21. **Run the protein gel on SDS-PAGE for about 50 minutes, then stain it with the staining solution.**

22. **Decolorization and photoresist**

After the protein gel is decolored with water and decolorizing solution, the gel is illuminutesated to observe the bands.

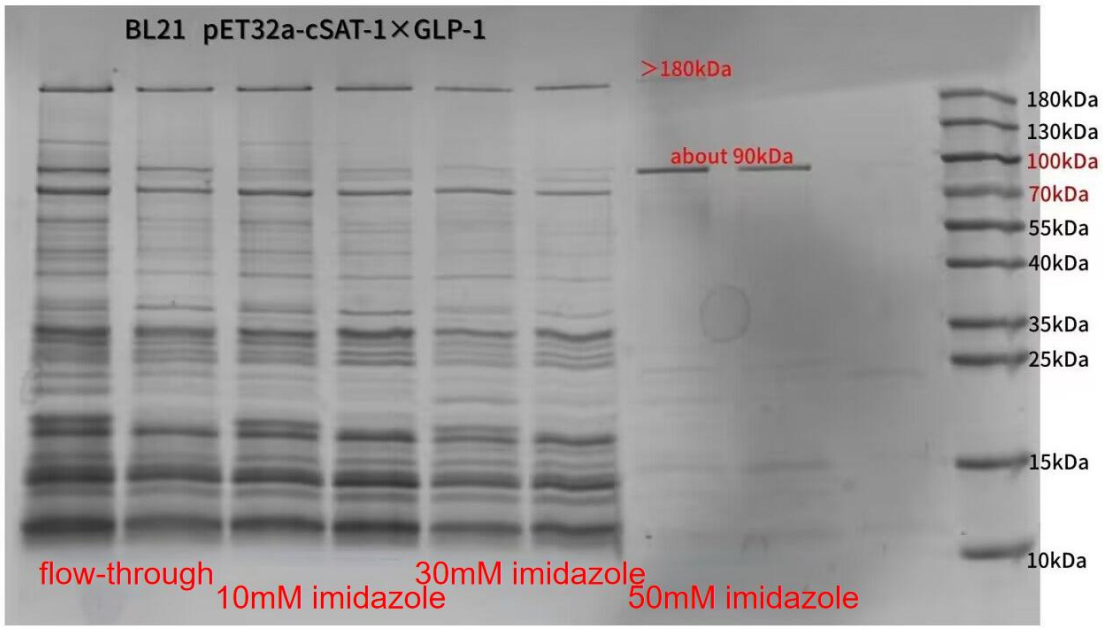

Figure 10 BL21 pET32a-cSAT-GLP-1 large-scale system

23. EcN (Nissle1917) with pET32a-His6 alone and EcN with pET32a-4GLP-1-5-LV are activated for bacterial culture. 1 mL of LA liquid medium is aspirated into sterilized 1.5 mL EP tubes, and then 9 μL of the bacterial liquid is aspirated into each EP tube. These are placed in a 37℃ shaker for overnight culture. A total of eight tubes are activated.

24. The activated EcN pET32a-4GLP1-5LV is respectively added to six 100 mL LB liquid media supplemented with Amp antibiotic. Around 11:00, they are placed in a 37℃, 200 rpm shaking incubator for a small-scale test. Three gradients of 0.6, 0.8, and 1.0 are set for OD600nm, and two gradients of 0.2 mM and 0.4 mM are set for the inducer IPTG, totaling six groups.

25. The EcN pET32a-His6 is activated. 10 μL of glycerol bacteria is aspirated into 1 mL of LA liquid culture meduim and placed in a 37℃ shaker for incubation at 11:18.

26. Six bottles of Nissle1917 pET32a-4GLP1-5LV are numbered A-F respectively, and the liquid of 1 mL bacteria is absorbed into the cuvette to measure OD600nm:

(1) Measurement time 13:10

Serial number	A	B	C	D	E	F
OD_{600nm}	0.289	0.299	0.331	0.297	0.253	0.268

The OD_{600nm} values do not reach the minutesimum gradient, so they are put back to 37℃ and cultured in 200 rpm shaker.

(2) Measurement time 13:42

Serial number	A	B	C	D	E	F
OD_{600nm}	0.641	0.628	0.672	0.663	0.566	0.602

The bacterial liquid from the conical bottles numbered E and F are selected as the OD_{600nm}=0.6 gradient samples, and numbered 1 and 2, respectively. The other four bottles are put back to 37℃ and cultured in 200 rpm shaker.

(3) Measurement time 14:08

Serial number	A	B	C	D
OD_{600nm}	0.813	0.816	0.871	0.839

The bacterial liquid from the conical flask numbered A and B are selected as OD_{600nm}=0.8 gradient samples and numbered 3 and 4, respectively. The other two bottles are put back to 37℃ and continued to be cultured in 200 rpm shaker.

(4) Measurement time 14:20

Aspirate 500 μL of the bacterial liquid into a cuvette filled with 500 μL of sterile water. The OD_{600nm} is measured after diluting the bacterial liquid twice.

Serial number	C	D
OD_{600nm}	0.597	0.580

Then the original OD_{600nm}=0.597 × 2 × 1.194 ,original OD_{600nm}=0.580 × 2 × 1.16 of bottle D is taken as the sample of OD_{600nm}=1.0 gradient and numbered 5 and 6 respectively.

Prepare the IPTG solution of 1 mol/L, and add the 2.383 g IPTG powder with sterile water or pure water to 10 mL, filtered and sterilized in the super-clean bench, then stored at -20℃ away from light.

27. Induced expression

Six bottles of bacterial liquid A, B, C, D, E and F are put into a shaker at 18℃ to cool down, and then in the super-clean bench, each bottle absorbed 1 mL into the cryotube as a control before induction, marked, and then added inducer IPTG to the six bottles of bacterial solution, tied the bacterial solution before induction to the corresponding conical bottle, put it into 18℃, 200 rpm shaker to induce expression for 18 hours:

Serial number	IPTG gradient	OD_{600nm} gradient	Serial number	IPTG gradient	OD_{600nm} gradient
①	0.2 mM	0.6	④	0.4 mM	0.8
②	0.4 mM	0.6	⑤	0.2 mM	1.0
③	0.2 mM	0.8	⑥	0.4 mM	1.0

28. Control

At 16:15, the OD_{600nm} gradient of the control liquid is 0.741, the range is 0.6-0.8, which meets the requirements. After cooling in a shaker at 18℃, 1 mL is absorbed into the cryotube in the super-clean bench as the control before induction, and then 0.4 mM IPTG is added to the bacterial liquid. The bacterial solution before induction is tied to a conical bottle, and the expression is induced by 200 rpm shaker at 18℃ for 18 hours.

29. In the super-clean bench, six bottles of bacteria No.123456 and control No.0 each absorbed 200 μL into the cryotube as the control after induction.

30. Collect bacteria

Each bottle of bacteria is collected with 50 mL centrifuge tube, centrifuge 4000 rpm , 10 minutes. leveling before centrifugation.

31. Resuspend

The strains collected after centrifugation are resuscitated with 10 mL buffer. Buffer formula: 20 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.5.

32. Break bacteria

The re-suspended bacterial liquid is put into the sample mouth of the germicidal machine to be broken according to the steps, and each tube is broken three times.

33. Centrifuge

The broken bacterial samples are injected 2 mL into the 2.0 mL EP tube, and two parallel tubes are made for each bacterial sample. 12000 rpm centrifuge for 30 minutes, then put the sample in a refrigerator at 4℃.

34. **Prepare protein gel** Prepare two plates of 15-well protein gel and one plate of 10-well protein gel.

35. Sample preparation (loading is 5 ×, abbreviated to 5 ×)

(1) Supernatant: 16 μL supernatant + 4 μL 5 ×.

(2) Precipitation: each tube of precipitation is re-suspended with 200 μL pure water, and the mixture after resuspension is 10 μL+4 μL 5 × + 6 μL pure water.

(3) The bacterial solution before induction : 16 μL + 4 μL 5 ×.

(4) The bacterial liquid after induction :16 μL + 4 μL 5 ×.

(5) The sample is boiled in a metal bath at 100℃ above 20 minutes.

36. Load the sample

(1) First plate protein gel (10-well):

marker 0-supernatant 0-precipitation 0-before induction, 0-after induction (control)

(2) second plate protein gel (15-well):

marker 1-supernatant 1-precipitation 1-before induction 1-after induction 2-supernatant 2-precipitation 2-before induction 2-after induction 3-supernatant 3-precipitation 3-before induction 3-after induction

(3) third plate protein gel (15-well):

4-supernatant 4-precipitation 4-before induction 4-after induction marker 5-supernatant 5-precipitation 5-before induction 5-after induction 6-supernatant 6-precipitation 6-before induction 6-after induction

37. Run SDS-PAGE about 50 minutes, and then dye.

38. Decolorization,photoresist

Gel imaging after the protein gel is decolorized with water and decolorizing solution.

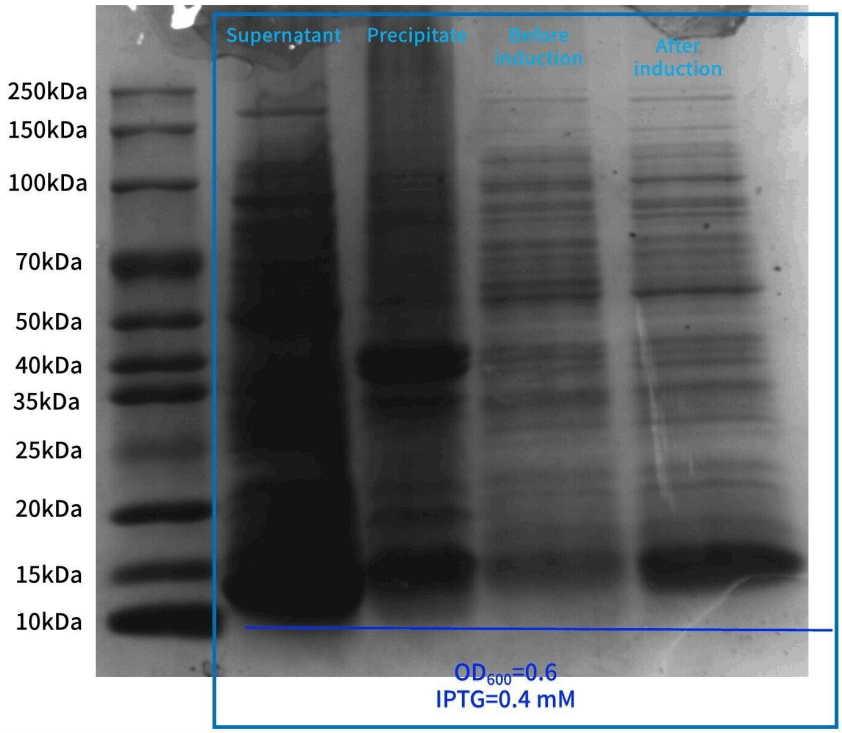

Figure 11 No.0 control

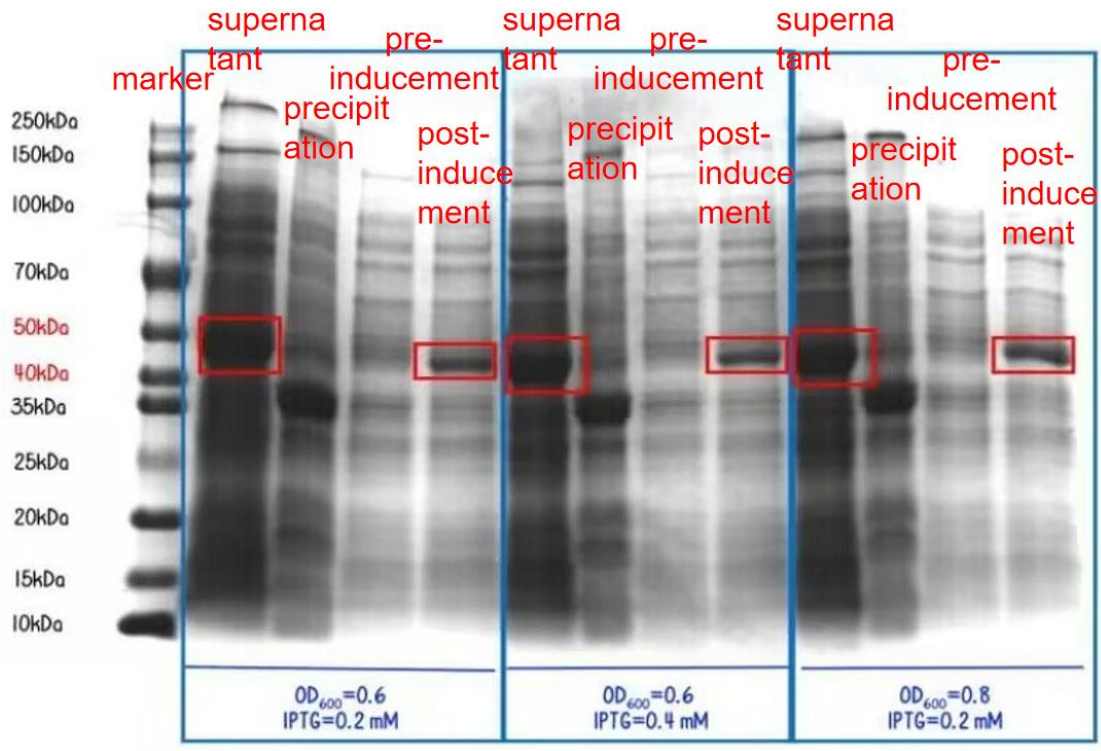

Figure 12 Samples 1,2 and 3

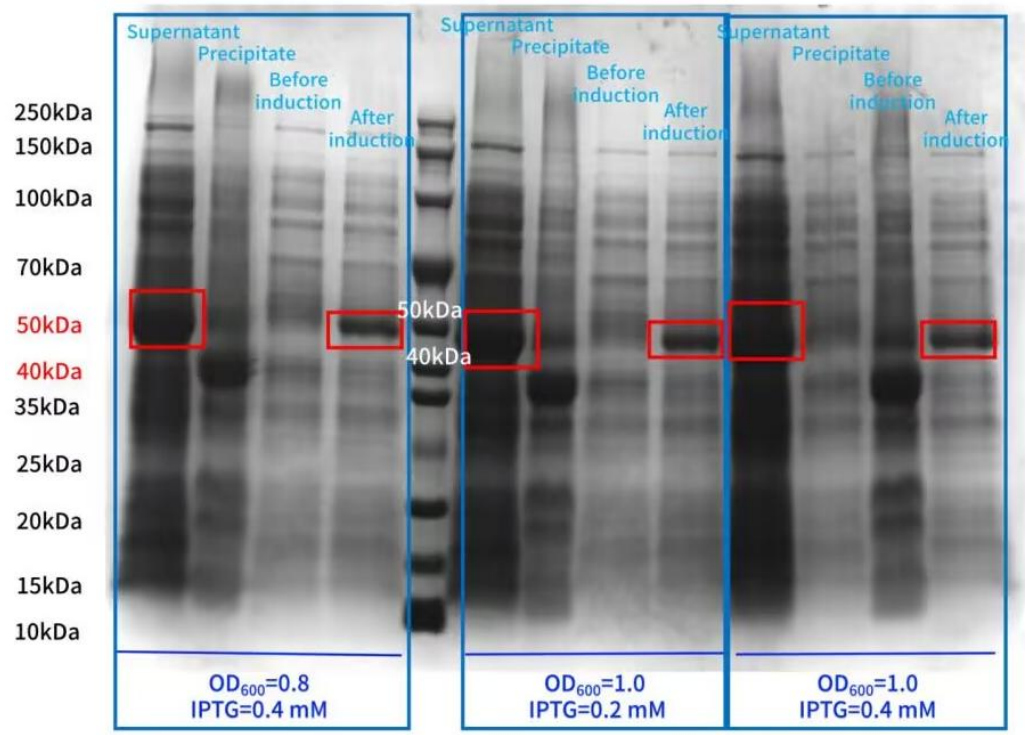

Figure 13 Samples 4,5 and 6

39. Activation of Glycerol preserved bacteria

ZM4 pTZ28a-His6-sfGFP, ZM4 pTZ28a-4GLP-1-5LV, ZMNP pTZ28a-sfGFP and ZMNP pTZ28a-4GLP-1-5LV glycerol preservation bacteria are activated. 6 μL of the glycerol preserved bacteria are absorbed in the clean bench and injected into 5mL of the RK liquid culture medium, and then cultured in a shaker at 30℃ for overnight.

40. **Activated glycerol ZM4 pTZ28a-His6-sfGFP, ZM4 pTZ28a-4GLP-1-5LV, ZMNP pTZ28a-His6-sfGFP, ZMNP pTZ28a-4GLP-1-5LV transfer system (250 mL cone flask with 100 mL RK liquid culture meduim). The activated bacterial liquid is poured into the large system and OD600nm is measured. In the super-clean bench, absorb 900 μL pure water into the colorimetric dish, then absorb the 100 μL bacteria liquid into the colorimetric dish and mix well, dilute it ten times. The amount of bacterial liquid that needs to be absorbed into the large system is calculated, so that the initial OD600nm is controlled at 0.1. 1 mL arabinose and 1.6 μL tetracycline are added to induce epida, and the mixed solution of 200 μL is absorbed into the EP tube of 2.0 mL to prepare for flow cytometry.**

41. **ZMNP Flow**

	First flow		Second flow		Thrid flow	
Time	2024.9.6 19：20		2024.9.7 10：40		2024.9.7 19：20	
Bacteria species	ZMNP pTZ28a-His6-sfGFP	ZMNP pTZ28a-4GLP-1-5LV	ZMNP pTZ28a-His6-sfGFP	ZMNP pTZ28a-4GLP-1-5LV	ZMNP pTZ28a-His6-sfGFP	ZMNP pTZ28a-4GLP-1-5LV
OD_{600nm}	0.43	0.73	3.22	3.78	5.35	5.02
	Fourth flow					
Time	2024.9.8 19：30					
Bacteria species	ZMNP pTZ28a-H	ZMNP pTZ28a-4				

	is6-sfGFP	GLP-1-5LV				
OD_{600nm}	5.39	4.91				

42. ZM4 Flow

	First flow		Second flow		Thrid flow	
Time	2024.9.7 19：20		2024.9.8 15：53		2024.9.9 10：20	
Bacteria species	ZM4 pTZ28a-His6-sfGFP	ZM4 pTZ28a-4GLP-1-5LV	ZM4 pTZ28a-His6-sfGFP	ZM4 pTZ28a-4GLP-1-5LV	ZM4 pTZ28a-His6-sfGFP	ZM4 pTZ28a-4GLP-1-5LV
OD_{600nm}	0.37	0.146	1.91	1.41	4.46	4.87
	Fourth flow					
Time	2024.9.10 9：35					
Bacteria species	ZM4 pTZ28a-His6-His6-sfGFP	ZM4 pTZ28a-4GLP-1-5LV				
OD_{600nm}	4.86	4.83				

43. ZMNP empty vector, ZMNP pTZ28a-4GLP-1-5LV collecting, breaking bacteria, sample preparation, running protein gel .

(1) Collect bacteria:Centrifuge at 4000 rpm at 4℃, centrifuge for 10 minutes, then pour out the supernatant, centrifuge with bacteria, repeat this step until all the bacteria have been collected.

(2) Resuspend: The strains collected in the centrifuge tube are resuscitated with 10

mL buffer. Buffer formula: 20 mM Tris, 150 mM NaCl,20 mM imidazole, pH 7.5.

(3) Break bacteria: After centrifugation, the bacteria are re-suspended with buffer, and the re-suspended bacteria solution is put into the loading sample wells of the crushing machine to break up according to the steps, and each tube is broken three times.

(4) Centrifuge: The broken bacterial samples are injected 2 mL into the 2.0 mL EP tube , and two parallel tubes are made for each bacterial sample. 12000 rpm centrifuge for 30 minutes.

(5) Prepare protein gel: Prepare two plates of 10-well protein gel.

(6) Sample preparation (loading is 5×, abbreviated to 5×)

① Supernatant: 16 μL supernatant + 4 μL 5×.

② Precipitation: each tube of precipitation is resuspended with 200μL pure water, and the mixture after resuspension is 10 μL+4 μL 5× + 6 μL pure water.

③ The bacterial solution before induction : 16 μL + 4 μL 5×.

④ The bacterial liquid after induction :16 μL + 4 μL 5×.

⑤ The sample is boiled in a metal bath at 100℃ above 20 minutes.

(7) Load the sample

① ZMNP empty vector:

supernatant, marker, precipitation, before induction, after induction

② ZMNP pTZ28a-4GLP-1-5LV

supernatant, precipitation, before induction, after induction, marker

44. Run SDS-PAGE about 38 minutes, and then dye with dye solution.

Gel imaging after the protein gel is decolorized with water and decolorizing solution.

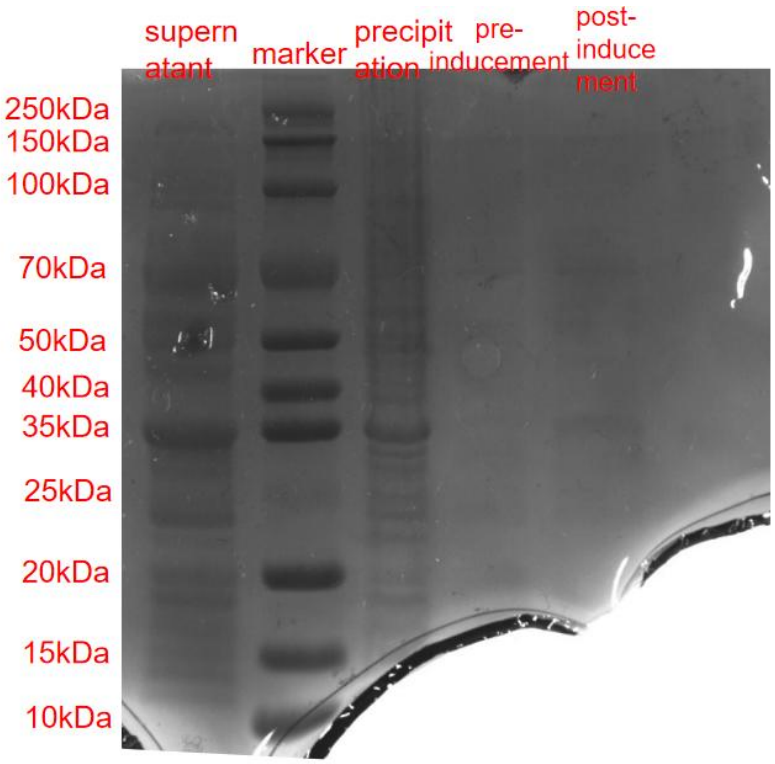

Figure 14 ZMNP empty vector

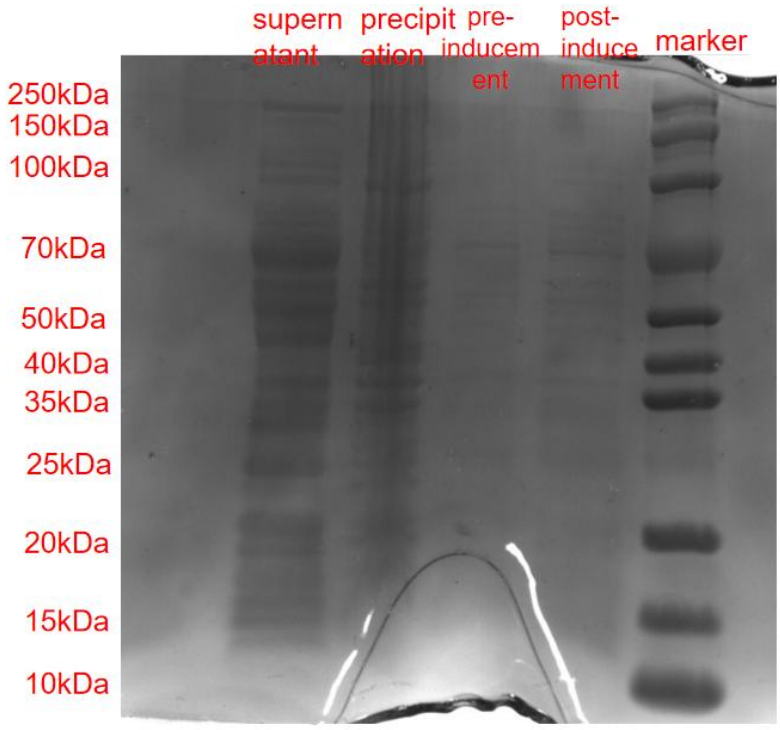

Figure 15 ZMNP pET32a-4GLP-1-5LV

45. **1 L buffer: 20 mM Tris and 150mM NaCl,20 mM imidazole solution are prepared with pH of 7.5.**

46. ZM4 empty vector, ZM4 pTZ28a-4GLP-1-5LV to collect bacteria, break bacteria, purify and incubate, pass through column,sample preparation, run protein gel.

(1) Collect bacteria: Centrifuge, 4000 rpm, 4℃, centrifuge for 10 minutes, then pour out the supernatant, add bacteria to centrifuge, repeat this step until all the bacteria have been collected.

(2) Resuspend: The strains collected in the centrifuge tube are resuscitated with 15 mL buffer. Buffer formula: 20 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.5.

(3) Break bacteria: After centrifugation, the bacteria are re-suspended with buffer, and the re-suspended bacteria solution is put into the sample mouth of the crushing machine to break up according to the steps, and each tube is broken three times.

(4) Centrifuge: The broken bacterial samples are injected 2 mL into the 2.0 mL EP tube, and two parallel tubes are made for each bacterial sample. 12000 rpm centrifuge for 30 minutes.

(5) Prepare protein gel: Prepare a plate of 10-well protein gel and a plate of 15-well protein gel.

(6) Sample preparation: The sample is boiled in a metal bath at 100℃ above 20 minutes.

(7) Load the sample

① ZM4 empty vector: marker, supernatant, precipitation, before induction, after induction

② ZM4 pTZ28a-4GLP-1-5LV：supernatant, precipitation, marker, flow-through, 20mM, 50mM, 75mM, 100mM, 200mM, 300mM

47. Run SDS-PAGE about 35 minutes, and then dye with dye solution

Gel imaging after the protein gel is decolorized with water and decolorizing solution.

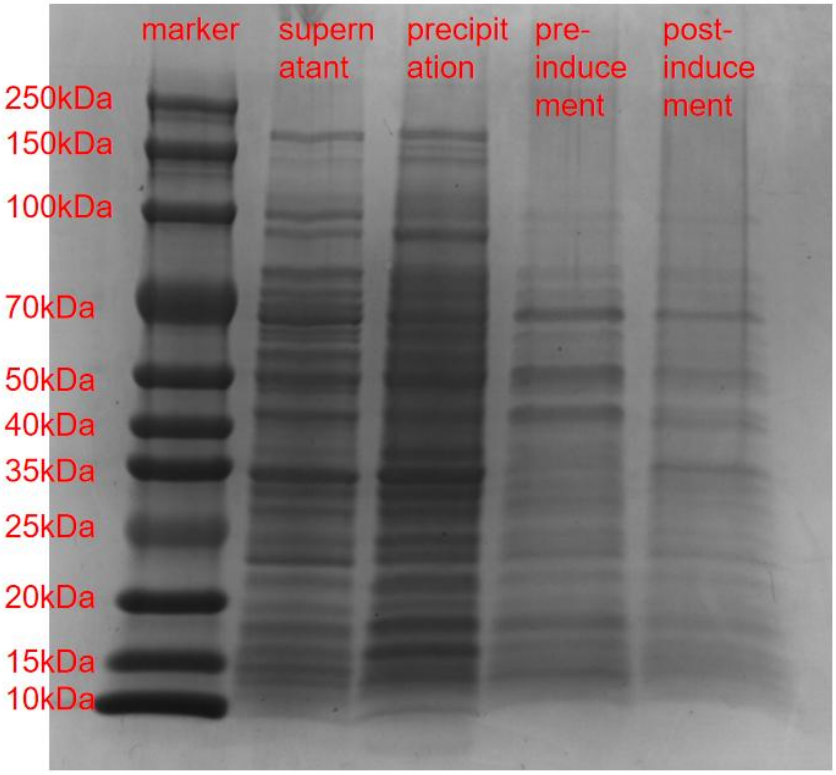

Figure 16 ZM4 empty vector

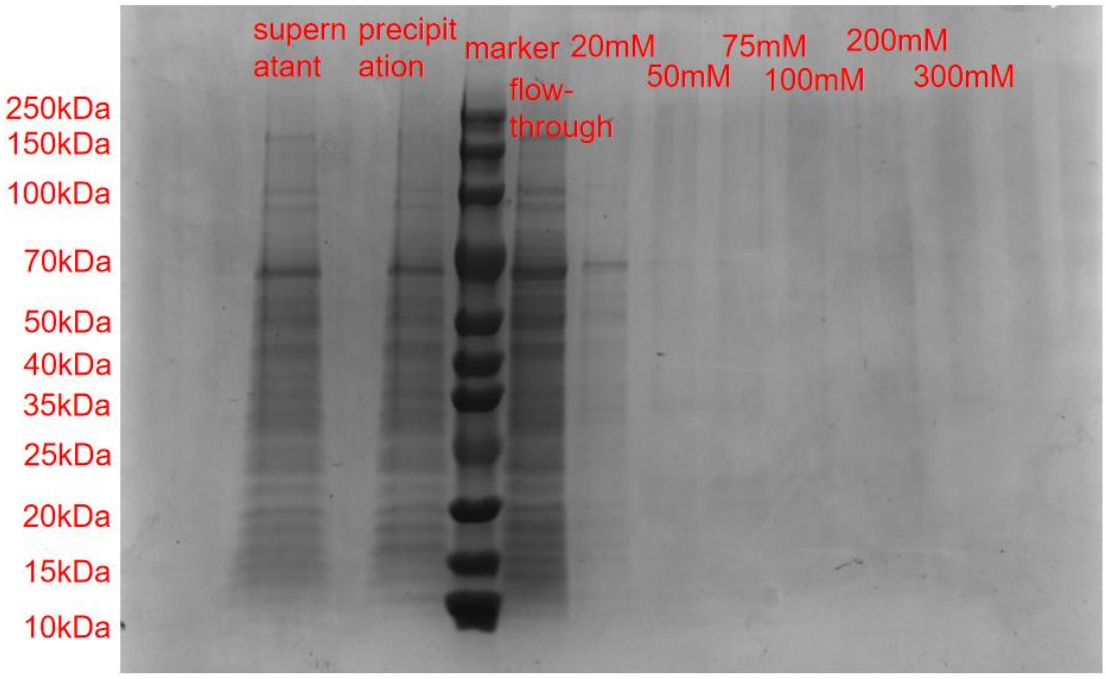

Figure 17 ZM4 pTZ28a-4GLP-1-5LV

48. Activation of Glycerol preserved bacteria

ZMNP pTZ28a-His6-sfGFP, ZMNP pTZ28a-4GLP-1-5LV glycerol preservation bacteria are activated. 6 μL of the glycerol preserved bacteria are absorbed and injected into 5 mL of the RK liquid culture medium in the clean bench , and then cultured in a shaker at 30℃ for overnight.

49. Transfer the activated glycerol ZMNP pTZ28a-His6-sfGFP, ZMNP pTZ28a-4GLP-1-5LV to small-scale system (containing 50 mL RK liquid culture meduim), and put it into a shaker at 30℃ to culture.

50. Activation of Glycerol preserved bacteria

EcN pET32a-His6, EcN pET32a-4GLP-1-5LV glycerol preservation bacteria are activated. 6 μL of the glycerol preserved bacteria are absorbed and injected into 5 mL of the LA liquid culture medium in the clean bench , and then cultured in a shaker at 30℃ for overnight.

51. Transfer ZMNP pTZ28a-His6-sfGFP, ZMNP pTZ28a-4GLP-1-5LV to a large system, and control the initial OD600nm is 0.8, add arabinose and tetracycline for induced expression, and put into a shaker at 30℃ to culture for 24 hours. Transfer EcN pET32a-His6, EcN pET32a-4GLP-1-5LV to a large system, put it into a shaker at 37℃ for culture, and get it out when the OD600nm is during 0.6-0.8, add the inducer IPTG for induction after cooling down to 18℃, and put into a shaker at 18℃ to culture for 16-20 hours.

52. Collect bacteria

Each bottle of bacteria is collected with 50 mL centrifuge tube, centrifuge 4000 rpm , 10 minutes. leveling before centrifugation.

53. Resuspend

The strains collected after centrifugation are resuscitated with 10 mL buffer. Buffer formula: 20 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.5.

54. Break bacteria

The re-suspended bacterial liquid is put into the sample mouth of the germicidal machine to be broken according to the steps, and each tube is broken three times.

55. Centrifuge

The broken bacterial samples are injected 2 mL into the 2.0 mL EP tube, and two parallel tubes are made for each bacterial sample. 12000 rpm centrifuge for 30 minutes, then put the sample in a refrigerator at 4℃.

56. **Prepare protein gel**

Prepare two plates of 15-well protein gel and one plate of 10-well protein gel.

57. Sample preparation

The sample is boiled in a metal bath at 100℃ above 20 minutes.

Load the sample

(1) ZMNP：

Marker supernatant of ZMNP empty vector precipitation of ZMNP empty vector supernatant of ZMNP pTZ28a-4GLP-1-5LV precipitation of ZMNP pTZ28a-4GLP-1-5LV flow-through 20mM imidazole 50mM imidazole 75mM imidazole 100mM imidazole 200mM imidazole 300mM imidazole 500mM imidazole

(2) EcN：

Supernatant of EcN pET32a-His6 precipitation of EcN pET32a-His6 supernatant of EcN pET32a-4GLP-1-5LV precipitation of EcN pET32a-4GLP-1-5LV flow-through 20mM imidazole 50mM imidazole 75mM imidazole 100mM imidazole 200mM imidazole 300mM imidazole 500mM imidazole

58. Protein gum SDS-PAGE about 35 minutes, and then dye with dye solution.

Gel imaging after the protein gel is decolorized with water and decolorizing solution.

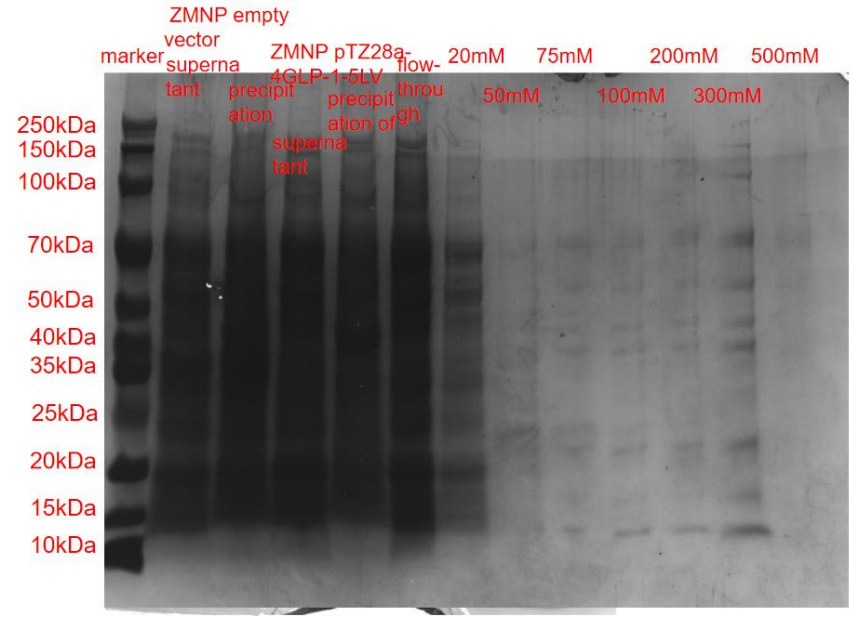

Figure 18 ZMNP

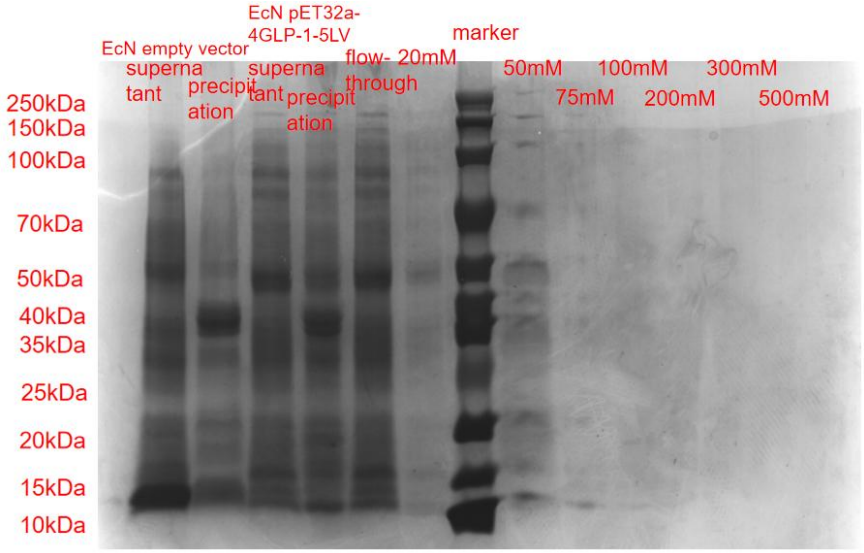

Figure 19 EcN

59. The first round of PCR

(1) Primers:

Piece 5×LV-F　　DNA：4GLP-1-5LV

Piece 5×LV-R　　size：240bp

Results: the concentration of plasmid fragment is 14.7 ng/μL.

(2) Primers:

Piece 4×GLP-1-F　　DNA：4GLP-1-5LV

Piece 4×GLP-1-R size：432bp

Results: the concentration of plasmid fragment is 31.15 ng/μL.

60. The second round of PCR

(1) Primers:

T5-4GLP-1-28aARM-F DNA:First round 4 4GLP-1

T5-4GLP-1-28aARM-R size：472bp

Results: the concentration of plasmid fragment is 22.45 ng/μL.

(2) Primers:

T5-4GLP-1-32a ARM-F DNA: First round 4 4GLP-1

T5-4GLP-1-32a ARM-R size：472bp

Results: the concentration of plasmid fragment is 2.9 ng/μL.

Primers:

T5-5LV-28a ARM-F DNA:first round 3 pieces of 5LV

T5-5LV-28a ARM-R size：280bp

Results: the concentration of the recovered plasmid fragment is 24.35 ng/μL.

(3) Primers:

T5-5LV-32a ARM-F DNA：first round 3 pieces of 5LV

T5-5LV-32a ARM-R size：280bp

Results: the concentration of plasmid fragment is 24.05 ng/μL.

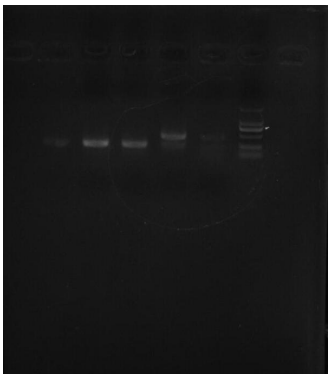

Figure 20

61. T5 enzyme link

Plasmid fragment 4GLP-1-32a arm and pET32a linear vector. According to the mole ratio fragment: vector ≥ 3:1, it is calculated that the fragment needs to be added 3.36 μL, vector needs to add 0.04 μL, water is added to 4 μL system, and then buffer and 0.5 μL of dilute released T5 enzyme are added. Under the condition of T5 enzyme link reaction, it is connected by T5 enzyme and transferred into DH5α competent state to form plasmid:

4GLP-1-32a ARM +pET32a —— pET32a-4GLP-1

Coated on the medium plate of LA in super-clean bench. It is cultured overnight in a constant temperature incubator at 37℃.

(1) Plasmid fragment 5LV-32a arm and pET32a linear vector.According to the mole ratio fragment: vector ≥ 3:1, it is calculated that the fragment needs to be added 3.1 μL, vector needs to add 0.1 μL, water is added to 4 μL system, and then buffer and diluted T5 enzyme 0.5μL are added. Under the condition of T5 enzyme reaction, it is connected by T5 enzyme and transferred into DH5α competent state to form plasmid:

5LV-32a ARM +pET32a —— pET32a-5LV

Coated on the medium plate of LA in the super-clean bench. It is cultured overnight in a constant temperature incubator at 37℃.

(2) Plasmid fragment 4GLP-1-28a Arm and pTZ28a linear vector.According to the mole ratio fragment: the vector ≥ 3:1, the calculated fragment needs to add 3.71 μL, the vector needs to add 0.07 μL, water is added to the 4 μL system, and then buffer and each 0.5 μL of the dilute released T5 enzyme are added. Under the condition of T5 enzyme link reaction, the fragment is connected by T5 enzyme and transferred into the DH5a competent state to form plasmid:

4GLP-1-28a ARM +pTZ28a —— pTZ28a-4GLP-1

coated on the medium plate of LK in the super-clean bench and cultured overnight in a constant temperature incubator at 37℃.

(3) Plasmid fragment 5LV-28a arm and pTZ28a linear vector. According to the mole ratio fragment: the carrier ≥ 3:1, the calculated fragment needs to add 3.95 μL, the vector needs to add 0.05 μL, and then buffer and diluted T5 enzyme 0.5 μL are added. Under the condition of T5 enzyme link reaction, it is connected by T5 enzyme and transferred into the DH5α competent state to form plasmid:

5LV-28a Arm + pTZ28a —— pTZ28a-5LV

spread on the culture medium plate of LK in the super-clean bench and cultured overnight in 37℃ constant temperature incubator.

62. After the transformed board is verified by monoclonal colony PCR, the correct bacteria are absorbed into the corresponding liquid culture meduim and cultured in a shaker at 37℃. DH5α pET32a-5LV strain, DH5α pET32a-4GLP-1 strain, DH5a pTZ28a-5LV strain and DH5a pTZ28a-4GLP-1 strain are obtained.

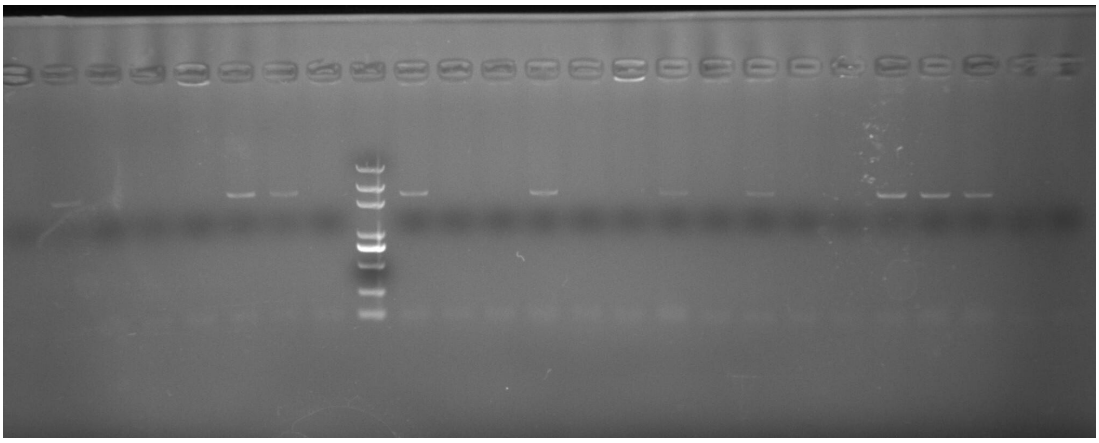

Figure 21

63. The cultured strains absorbed 60% of glycerol and 1 mL respectively in the super-clean bench into the frozen tube and stored in the refrigerator at-80℃. At the same time, 1 mL bacterial liquid is absorbed into the 1.5 mL EP tube and sent to the biological company for sequencing to further verify the correctness of the transformants.

64. Plasmid extraction

Extraction of plasmids from cultured DH5α pTZ28a-5LV and DH5α pTZ28a-4GLP-1 strains.

(1) DH5α pTZ28a-5LV The concentration of plasmid extracted: 90.35 ng/μL.

(2) DH5α pTZ28a-4GLP-1 The concentration of plasmid extracted: 23.95 ng/μL.

65. Electroporation

PTZ28a-5LV and pTZ28a-4GLP-1 plasmids are transferred into ZMNP-T7 and ZM4-T7 competent cells and transformed into one tube each. After electric transfer, ZMNP-T7 and ZM4-T7 are cultured in a shaker at 30℃ for 4-6 hours, then coated on the culture medium plate of RK in super-clean bench, sealed with a sealing film, and then put into a constant temperature incubator at 30℃ for overnight culture.

66. Monoclonal colony PCR

(1) Primers:

T5-5LV-28aw-F　　DNA：ZM4-T7 pTZ28a-5LV

test 32a-cSAT-GLP-R　　size：4198bp

Results: there are bands in agarose gel electrophoresis and the size is correct.

(2) Primers:

T5-4GLP-1-28aw-F　　DNA：ZM4-T7 pTZ28a-4GLP-1

test 32a-cSAT-GLP-R　　size：4198bp

Results: there are bands in agarose gel electrophoresis and the size is correct.

(3) Primers:

T5-5LV-28aw-F　　DNA：ZMNP-T7 pTZ28a-5LV

test 32a-cSAT-GLP-R　　size：4198bp

Results: there are bands in agarose gel electrophoresis and the size is correct.

(4) Primers:

T5-4GLP-1-28aw-F　　DNA：ZMNP-T7 pTZ28a-4GLP-1

test 32a-cSAT-GLP-R　　size：4198bp

Results: there are bands in agarose gel electrophoresis and the size is correct.

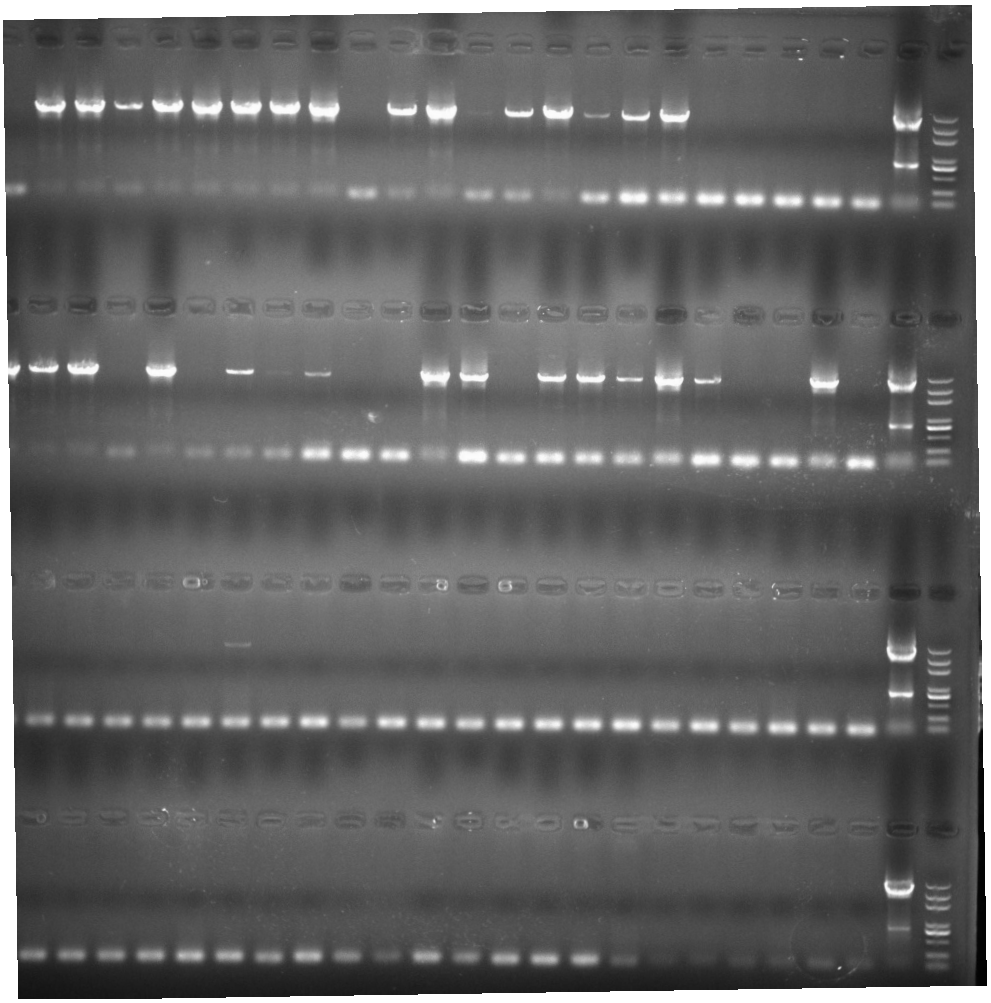

Figure 22

67. Culture of bacteria

Select the strain with correct transformation and inoculate it into the RK liquid culture meduim, followed by culturing in a shaker at 30℃. As a result, the ZM4-T7 pTZ28a-4GLP-1 strain, the ZM4-T7 pTZ28a-5LV strain, the ZMNP-T7 pTZ28a-4GLP-1 strain, and the ZMNP-T7 pTZ28a-5LV strain are obtained.

68. Preserve the bacteria and send it for sequencing.

Strains preserved in 60% glycerol：ZM4-T7 pTZ28a-4GLP-1 strain, ZM4-T7 pTZ28a-5LV strain, ZMNP-T7 pTZ28a-4GLP-1 strain, and ZMNP-T7 pTZ28a-5LV strain. Simultaneously, 1 mL of the bacterial liquid is respectively aspirated into 1.5 mL EP tubes and sent to a biological company for sequencing to further validate the correctness of the transformants.