**J23106-B0034-Endolysin-B0010-B0012-R0010-B0034-Holin-B0010-B0012**

**-J23118-B0034-NrtA-B0015**

:: NrtA C33 PCR (VF2, VR)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 62 |
| 10x KOD plus buffer | 10 |
| NrtA C33 plasmid | 4 |
| Primer VF2(20uM) | 4 |
| Primer VR(20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 4 |
| KOD plus polymerasae | 2 |
| Total | 100 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X30 |
| 48 | 30sec |
| 68 | 2min |
| 68 | 10min |  |
| 20 | - |  |

:: Run gel

100mV, 25min

The expected length of NrtA C33: 1805bp.

Cut gel and purified (elute with 15ul ddH2O)

(See “Gel Extraction Protocol Procedure” of “GenepHlowTM Gel/PCR Kit”)

:: Digest

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| NrtA C33 | 15 |
| 10X Cutsmart buffer | 2 |
| EcoRI | 1.5 |
| SpeI | 1.5 |
| Total | 20 |

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Endolysin-Holin (backbone) | 30 |
| 10X Cutsmart buffer | 4 |
| EcoRI | 3 |
| XbaI | 3 |
| Total | 40 |

Incubate at 37°C for 1 hr.
Run gel to check concentration (1:1, 100mV, 25min).

If the concentration is too low, digest overnight.

The expected length of NrtA C33 digest product: 1554bp.

The expected length of Endolysin-Holin digest product: 3820bp.

Put at 65°C for 20 min.

:: Ligation

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| 10X Rapid Ligation Buffer, T4 DNA Ligase | 1 |
| NrtA C33 (insert) | \* |
| Endolysin-Holin (backbone) | \* |
| T4 DNA Ligase (3 Weiss units/μl) | 1 |
| Total | 10 |

\* Run gel (1:1, 100mV, 25min), see concentration

Calculate the amount of backbone and insert (use 7:1) <http://nebiocalculator.neb.com/#!/ligation>

Put at 25°C for 1hr.

:: Transformation

1. Put 5ul ligation sample and 100ul competent cell (the structure is very fragile, don’t spin down) into eppendorf, vortex for 1sec. This step must be done on ice.
2. Put on ice for 30 min.
3. Heat shock: 41℃ for 1 min.
4. Put on ice for 10~30 min.
5. Add 873ul LB liquid and 27ul 30% glucose (that is, LB with 0.9% glucose) to repair the cell wall.
6. Culture in the 37℃ incubator for 1hr at 200k rpm.
7. Centrifuge for 2 min at 3.4k rpm.
8. Take away 950ul.
9. Spread the plate in the hood:
Plate: LB agar plate with 0.9% glucose + Chloramphenicol (CM)
	1. Dry the plate in the hood for 20min.
	2. Pipetting 50ul and spread on the plate.
	3. Label: name, date, plasmid backbone, part/circuit, type of plate
10. Incubate at 37℃ for 12~16 hr.

:: Liquid culture

1. Pick the single colony and mark on the plate, you incubate the day before.
2. For each centrifuge tube, the components of liquid culture are as following.

|  |  |
| --- | --- |
| Liquid Culture Component | Volume |
| LB liquid | 5 ml |
| Chloramphenicol (CM) | 5 ul |
| 30% glucose | 150 ul |
| Total | 5 ml |

1. Using a sterile pipette tip, select a single colony from the plate you mark.
2. Drop the tip into the liquid culture.
3. Loosely cover the liquid culture with the cap.
4. Incubate bacterial liquid culture at 37℃ for 12~16 hr and at 200krpm.

:: Plasmid extraction

See protocol of “PrestoTM Mini Plasmid Kit”.

(In the last step, elution, add 30~50 ul of water into the column.)

:: RE check

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Endolysin-Holin-NrtAC33 plasmid | 2 |
| 10X Cutsmart buffer | 1 |
| ddH2O | 6.5 |
| XmaI | 0.5 |
| Total | 10 |

1. Put at 37°C for 1hr, and then put at 65°C for 20 min.
2. Run gel (1:5, 100mV, and 25 min) to check length.
The expected length is 558bp and 4816bp.