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

:: Holin PCR (VF2, VR)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 62 |
| 10x KOD plus buffer | 10 |
| Holin 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 | 1min 30sec |
| 68 | 10min |  |
| 20 | - |  |

:: Run gel

100mV, 25min

Cut gel and purified (elute with 15ul ddH2O)

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

:: Digest

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

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Endolysin (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 Holin digest product: 1068bp.

The expected length of Endolysin digest product: 2767bp.

Put at 65°C for 20 min.

:: Ligation

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| 10X Rapid Ligation Buffer, T4 DNA Ligase | 1 |
| Holin digest product | \* |
| Endolysin digest product | \* |
| 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.
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.

:: Colony PCR

1. Pick the single colony and mark.
2. Prepare PCR mix

|  |  |
| --- | --- |
| Reaction Component(each PCR tube) | Volume (ul) |
| ddH2O | 7.96 |
| 10x Dream taq buffer | 1 |
| Primer P0020(20uM) | 0.4 |
| Primer P0021(20uM) | 0.4 |
| dNTP (10mM) | 0.2 |
| Dream taq | 0.04 |
| Total | 10 |

1. Streak out every single colony on second plate (LBA with 0.9% glucose + CM) and mark, and then pipette in PCR mix.
2. Run PCR

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2 min |  |
| 94 | 20 sec | X30 |
| 44.7 | 30 sec |
| 72 | 40sec |
| 72 | 10 min |  |
| 20 | - |  |

1. Run gel (1:5, 100mV, 25 min) to check whether the colony is right.

The expected length is 657bp (Holin).