**R0010-B0034-Holin-B0010-B0012**

:: PCR

Tube E　　pSB1C3-J04450 (backbone)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 96 |
| 10x KOD plus buffer | 15 |
| pSB1C3-J04450 | 3 |
| Primer P0012(20uM) | 6 |
| Primer P0017(20uM) | 6 |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD plus polymerasae | 3 |
| Total | 150 |

Tube F

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 96 |
| 10x KOD plus buffer | 15 |
| pSB1C3-J04450 | 3 |
| Primer P0011(20uM) | 6 |
| Primer P0018(20uM) | 6 |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD plus polymerasae | 3 |
| Total | 150 |

Tube G BBa\_K112000 (Holin)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 96 |
| 10x KOD plus buffer | 15 |
| BBa\_K112000 | 3 |
| Primer P0021(20uM) | 6 |
| Primer P0019(20uM) | 6 |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD plus polymerasae | 3 |
| Total | 150 |

Tube H

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 96 |
| 10x KOD plus buffer | 15 |
| BBa\_K112000 | 3 |
| Primer P0022(20uM) | 6 |
| Primer P0020(20uM) | 6 |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD plus polymerasae | 3 |
| Total | 150 |

Tube E, F

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2 min |  |
| 94 | 20 sec | X30 |
| 40.6 | 30 sec |
| 68 | 3 min |
| 68 | 10 min |  |
| 20 | - |  |

Tube G, H

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X30 |
| 31.5 | 30sec |
| 68 | 1min |
| 68 | 10min |  |
| 20 | - |  |

:: Run gel

Tube G, H: 100mV, 32min

Tube E, F: 100mV, 38min

Cut gel and purified (elute with 15ul ddH2O)

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

EF, GH elute together

:: Annealing

E+F (EF elute together)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Tube E+F | 15 |
| 10X KOD plus buffer | 2 |
| ddH2O | 3 |
| Total | 20 |

 G+H (GH elute together)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Tube G+H | 15 |
| 10X KOD plus buffer | 2 |
| ddH2O | 3 |
| Total | 20 |

Annealing

|  |  |
| --- | --- |
| Reaction Temperature | Time |
| 95 | 4min |
| 25 | 20min |
| 20 | - |

:: DpnI (Tube E+F)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Annealing product (E+F) | 20 |
| 10X Cutsmart buffer | 2.5 |
| ddH2O | 1 |
| DpnI | 1.5 |
| Total | 25 |

Incubate in 37°C 1hr, then 65°C 20 min.

:: PNK (Tube G+H)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Annealing product (G+H) | 20 |
| ATP | 2.5 |
| 10X PNK buffer | 2.5 |
| PNK | 1 |
| Total | 26 |

Put at 37°C for 1hr, then at 65°C for 20 min.

:: Ligation

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| 10X Rapid Ligation Buffer, T4 DNA Ligase | 1 |
| pSB1C3-J04450 (backbone) Tube E+F | \* |
| BBa\_K112000 (Holin) Tube G+H | \* |
| 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 45 sec.
4. Put on ice for 10 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 | 3 min |
| 72 | 10 min |  |
| 20 | - |  |

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