**NrtA**

:: Annealing: Promoter + RBS

Tube 1 (Promoter + RBS)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 7 |
| Primer P0001(20uM) | 1 |
| Primer P0002(20uM) | 1 |
| 10x KOD plus buffer | 1 |
| Total | 10 |

Annealing

|  |  |
| --- | --- |
| Reaction Temperature (°C) | Time |
| 95 | 4min |
| 25 | 20min |
| 20 | - |

:: PNK: Tube 1

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Tube 1 annealing product | 10 |
| ddH2O | 5 |
| 10X PNK buffer | 2 |
| ATP | 2 |
| PNK | 1 |
| Total | 20 |

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

:: Backbone digestion

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| pSB1C3-J04450 | 30 |
| 10X Fast Digest Green Buffer | 4 |
| EcoRI | 3 |
| PstI | 3 |
| Total | 40 |

37°C for 30min

65°C for 15min

Run gel: 1:5, 100mV, 30min

Cut gel (the 2029bp band) and purified (elute with 15ul ddH2O)

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

:: PCR: NrtA + Terminator

Tube 2

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 60 |
| 10x KOD Hot Start Buffer | 10 |
| PCC6803 gDNA (50ng) | 4 |
| Primer P0003(20uM) | 4 |
| Primer P0006(20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 6 |
| KOD Hot Start polymerasae | 2 |
| Total | 100 |

Tube 3

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 60 |
| 10x KOD Hot Start Buffer | 10 |
| PCC6803 gDNA (50ng) | 4 |
| Primer P0004(20uM) | 4 |
| Primer P0006(20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 6 |
| KOD Hot Start polymerasae | 2 |
| Total | 100 |

Tube 4

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 60 |
| 10x KOD Hot Start Buffer | 10 |
| pSB1C3-B0015 | 4 |
| Primer P0005(20uM) | 4 |
| Primer P0007(20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 6 |
| KOD Hot Start polymerasae | 2 |
| Total | 100 |

Tube 5

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 60 |
| 10x KOD Hot Start Buffer | 10 |
| pSB1C3-B0015 | 4 |
| Primer P0008(20uM) | 4 |
| Primer P0006(20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 6 |
| KOD Hot Start polymerasae | 2 |
| Total | 100 |

Tube 2, 3, 4, 5

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 2 min |  |
| 95 | 20 sec | X30 |
| 33.6 | 30 sec |
| 70 | 2 min |
| 70 | 10 min |  |
| 20 | - |  |

Run gel (Tube 2~5) (1:5, 100mV, 25min)

Cut gel and purified (elute with 15ul ddH2O)

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

:: Fusion PCR: Tube A, Tube B

Tube A (Tube 2 + Tube 4)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 98 |
| 10x KOD Hot Start Buffer | 15 |
| Tube 2 | 0.5 |
| Tube 4 | 0.5 |
| Primer P0003(20uM) | 6 (add after run 5 cycles) |
| Primer P0007(20uM) | 6 (add after run 5 cycles) |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD Hot Start polymerasae | 3 |
| Total | 150 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2 min |  |
| 94 | 20 sec | X5 |
| 51 | 30 sec |
| 68 | 2 min |
| Pause and each PCR tube add 2ul Primer P0003, 2ul Primer P0007 | | |
| 94 | 20 sec | X30 |
| 53.2 | 30 sec |
| 68 | 2 min |
| 68 | 10 min |  |
| 20 | - |  |

Tube B (Tube 3 + Tube 5)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 98 |
| 10x KOD Hot Start Buffer | 15 |
| Tube 3 | 0.5 |
| Tube 5 | 0.5 |
| Primer P0004(20uM) | 6 (add after run 5 cycles) |
| Primer P0008(20uM) | 6 (add after run 5 cycles) |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD Hot Start polymerasae | 3 |
| Total | 150 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2 min |  |
| 94 | 20 sec | X5 |
| 51 | 30 sec |
| 68 | 2 min |
| Pause and each PCR tube add 2ul Primer P0004, 2ul Primer P0008 | | |
| 94 | 20 sec | X30 |
| 53.2 | 30 sec |
| 68 | 2 min |
| 68 | 10 min |  |
| 20 | - |  |

Run gel (Tube A, B) (1:5, 100mV, 25min)

Cut gel and purified (elute with 15ul ddH2O)

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

AB elute together

:: Annealing: Tube A+B

A+B (AB elute together)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Tube A+B | 15 |
| 10X KOD Hot Start Buffer | 2 |
| ddH2O | 3 |
| Total | 20 |

|  |  |
| --- | --- |
| Reaction Temperature (°C) | Time |
| 95 | 4 min |
| 25 | 15 min |
| 20 | - |

:: PNK: Tube A+B

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Tube A+B | 20 |
| 10X KOD plus buffer | 3 |
| ATP (10mM) | 3 |
| ddH2O | 3 |
| PNK | 1 |
| Total | 30 |

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

:: Ligation

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| 10X Rapid Ligation Buffer, T4 DNA Ligase | 1 |
| pSB1C3-J04450 (Backbone) | \* |
| Tube 1: Promoter + RBS (insert 1) | \* |
| Tube A+B: NrtA + Terminator (insert 2) | \* |
| 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 20 min.
5. Add 900ul LB liquid 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 + 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. Incubator 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 VF2 (20uM) | 0.4 |
| Primer VR (20uM) | 0.4 |
| dNTP (10mM) | 0.2 |
| Dream taq | 0.04 |
| Total | 10 |

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

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 2 min |  |
| 95 | 30 sec | X30 |
| 48 | 30 sec |
| 72 | 2 min |
| 72 | 10 min |  |
| 20 | - |  |

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

:: 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 |
| 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 200k rpm.

:: 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) |
| NrtA plasmid | 7 |
| 10X Fast Digest Buffer | 1 |
| HindIII | 1 |
| XhoI | 1 |
| Total | 10 |

1. Put at 37°C for 20hr, 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 892bp, 1540bp, and 1169bp.