**IndC**

Transformation K1152008 into compentent cell

Colony PCR Tube3,5,8

Tube1 (PCR of PrbcL)

P0023 AATTC TGATGGAAAAAGCACTGTAA

P0026 tgtgtaatattattttctaa CATGTCGTCTCTCCCTAGAG

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| 7942 gDNA | 1 |
| Primer P0023(20uM) | 1 |
| Primer P0026(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 47.87 | 30sec |
| 72 | 4 min30sec |
| 72 | 10min |  |
| 20 | - |  |

P0023 Tm: 52.87

P0026 Tm: 53.34

Tube 2

P0024: C TGATGGAAAAAGCACTGTAA

P0026: tgtgtaatattattttctaa CATGTCGTCTCTCCCTAGAG

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| 7942 gDNA | 1 |
| Primer P0024(20uM) | 1 |
| Primer P0026(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 47.87 | 30sec |
| 72 | 4 min30sec |
| 72 | 7min |  |
| 20 | - |  |

P0024 Tm: 52.87

P0026 Tm: 53.34

Tube 3

P0025: CTCTAGGGAGAGACGACATG ttagaaaataatattacaca

P0027: gttagattattttctcaatct

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| K1152008 | 1 |
| Primer P0025(20uM) | 1 |
| Primer P0027(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 33.88 | 30sec |
| 72 | 4 min30sec |
| 72 | 7min |  |
| 20 | - |  |

P0025 Tm: 38.88

P0027 Tm: 42

Tube 4

P0025: CTCTAGGGAGAGACGACATG ttagaaaataatattacaca

P0028: CCGGG ttagattattttctcaatct

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| K1152008 | 1 |
| Primer P0025(20uM) | 1 |
| Primer P0028(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 33.88 | 30sec |
| 72 | 4 min30sec |
| 72 | 7min |  |
| 20 | - |  |

P0025 Tm: 38.88

P0028 Tm: 42

All cut gel purified and dissolved in 15 ul ddH2O

Load 1 ul

|  |  |  |
| --- | --- | --- |
| RED Program19 /GRAY | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X5 |
| 51 | 30sec |
| 68 | 2min |
| Pause and add 2ul Pimer P0003, 2ul Primer P0007 | | |
| 94 | 20sec | X30 |
| 53.2 | 30sec |
| 68 | 2min |
| 68 | 10min |  |
| 20 | - |  |

Put tube 2. With tube 4. (tube B)

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 96 |
| 10x KOD hot start buffer | 15 |
| Tube 3 | Calculate ng 0.5 |
| Tube 5 | Calculate ng 0.5 |
| Primer P0024 (20uM) | 6 |
| Primer P0028 (20uM) | 6 |
| dNTP (2mM) | 15 |
| 25mM MgSO4 | 6 |
| KOD hot start polymerasae 1U/ul | 3 |
| Total | 150 |

DAY2

Tube1 (PCR of PrbcL)

P0023 AATTC TGATGGAAAAAGCACTGTAA

P0026 tgtgtaatattattttctaa CATGTCGTCTCTCCCTAGAG

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| 7942 gDNA | 2 |
| Primer P0023 (20uM) | 1 |
| Primer P0026 (20uM) | 1 |
| dNTP (2mM) | 2.5 |
| 25mM MgSO4 | 1.5 |
| KOD PLUS polymerasae 1U/ul | 0.5 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X30 |
| 47.87 | 30sec |
| 68 | 4 min30sec |
| 68 | 10min |  |
| 20 | - |  |

Tube 2

P0024: C TGATGGAAAAAGCACTGTAA

P0026: tgtgtaatattattttctaa CATGTCGTCTCTCCCTAGAG

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| 7942 gDNA | 2 |
| Primer P0024 (20uM) | 1 |
| Primer P0026 (20uM) | 1 |
| dNTP (2mM) | 2.5 |
| 25mM MgSO4 | 1.5 |
| KOD PLUS polymerasae 1U/ul | 0.5 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X30 |
| 47.87 | 30sec |
| 68 | 4 min30sec |
| 68 | 10min |  |
| 20 | - |  |

Tube 3

P0025: CTCTAGGGAGAGACGACATG ttagaaaataatattacaca

P0027: gttagattattttctcaatct

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| K1152008 | 2 |
| Primer P0025 (20uM) | 1 |
| Primer P0027 (20uM) | 1 |
| dNTP (2mM) | 2.5 |
| 25mM MgSO4 | 1.5 |
| KOD PLUS polymerasae 1U/ul | 0.5 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X30 |
| 33.88 | 30sec |
| 68 | 4 min30sec |
| 68 | 10min |  |
| 20 | - |  |

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 52 |
| 10x KOD PLUS buffer | 10 |
| K1152008 | 12 |
| Primer P0025 (20uM) | 4 |
| Primer P0027 (20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 6 |
| KOD PLUS polymerasae 1U/ul | 2 |
| Total | 100 |

Tube 4

P0025: CTCTAGGGAGAGACGACATG ttagaaaataatattacaca

P0028: CCGGG ttagattattttctcaatct

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 13 |
| 10x KOD PLUS buffer | 2.5 |
| K1152008 | 2 3 |
| Primer P0025 (20uM) | 1 |
| Primer P0028 (20uM) | 1 |
| dNTP (2mM) | 2.5 |
| 25mM MgSO4 | 1.5 |
| KOD PLUS polymerasae 1U/ul | 0.5 |
| Total | 25 |

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 52 |
| 10x KOD PLUS buffer | 10 |
| K1152008 | 12 |
| Primer P0025 (20uM) | 4 |
| Primer P0028 (20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 6 |
| KOD PLUS polymerasae 1U/ul | 2 |
| Total | 100 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X30 |
| 33.88 | 30sec |
| 68 | 4 min30sec |
| 68 | 10min |  |
| 20 | - |  |

0822:

Tube1~4 teplate add 2 ul

0823:

redo tube2，tube34 template add 3ul

redo again tube1,2 using all 7942 gDNA

Digest check: J04450-K112000 (Pst1,Sma1), J04450(Pst1),K112000(Sma1)

result: J04450-K112000 (Pst1,Sma1) only one band，J04450(Pst1) only one band，K112000(Sma1) didn’t has band

Digest check: K1152008 (for checking IndC gene sequence and length)

Fusion PCR

All cut gel purified and dissolved in 15 ul ddH2O

Load 1 ul for concentration check

Put tube 1. With tube 3. (Tube A)

|  |  |  |  |
| --- | --- | --- | --- |
| Reaction Component | Volume (ul) | 0825 | 0905 |
| ddH2O | 12 | 13.5 | 33.88 |
| 10x KOD PLUS buffer | 2.5 | 2.5 | 5 |
| Tube 1-1 | 0.5 | 1.5 | 0.12 |
| Tube indC 3 | 4 | 1.5 | 1 |
| Primer P0023 (20uM) | 1 | 1 | 2 |
| Primer P0027 (20uM) | 1 | 1 | 2 |
| dNTP (2mM) | 2.5 | 2.5 | 5 |
| 25mM MgSO4 | 1 | 1 | 2 |
| KOD PLUS polymerasae 1U/ul | 0.5 | 0.5 | 1 |
| Total | 25 | 25 | 50 |

Tube 1-1 and Tube indC 3 has same concentration

Mole: 1:0.12

P0023 Tm: 59.09

P0027 Tm: 43.51

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X5 |
| 51 | 30sec |
| 68 | 5min |
| Pause and add 2ul Pimer P0023, 2ul Primer P0027 | | |
| 94 | 20sec | X30 |
| 33.51 | 30sec |
| 68 | 5min |
| 68 | 10min |  |
| 20 | - |  |

51: overlap sequence Tm

Put tube 2. With tube 4. (tube B)

|  |  |  |  |
| --- | --- | --- | --- |
| Reaction Component | Volume (ul) | 0825 | 0905 |
| ddH2O | 10 | 13.5 | 33.88 |
| 10x KOD PLUS buffer | 2.5 | 2.5 | 5 |
| Tube 2-1 | 0.5 | 1.5 | 0.12 |
| Tube indC4 | 6 | 1.5 | 1 |
| Primer P0024 (20uM) | 1 | 1 | 2 |
| Primer P0028 (20uM) | 1 | 1 | 2 |
| dNTP (2mM) | 2.5 | 2.5 | 5 |
| 25mM MgSO4 | 1 | 1 | 2 |
| KOD PLUS polymerasae 1U/ul | 0.5 | 0.5 | 1 |
| Total | 25 | 25 | 50 |

Tube 2-1 and Tube indC4 has same concentration

P0024 Tm: 54.16

P0028 Tm: 58.86

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 94 | 2min |  |
| 94 | 20sec | X5 |
| 51 | 30sec |
| 68 | 5min |
| Pause and add 2ul Pimer P0024, 2ul Primer P0028 | | |
| 94 | 20sec | X30 |
| 44.16 | 30sec |
| 68 | 5min |
| 68 | 10min |  |
| 20 | - |  |

All cut gel and purified, dissolve in 15ul ddH2O sequentially, totally A+B=15 ul

A+B

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| A+B | 15 |
| 10X PCR buffer | 2 |
| ddH2O | 3 |
| Total | 20 |

Annealing

|  |  |
| --- | --- |
| Reaction Temperature | Time |
| 95°c | 4min |
| 25°c | 15min ref. [1] |
| 20°c | - |

Backbone digestion

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| Cut smart buffer | 2.5 |
| backbone | 19.5 |
| EcoR1-HF | 1.5 |
| Xma1 | 1.5 |
| total | 25 |

:: Cloning & ligation

Run gel check concentration

IndC+Prbcl:4263

Backbone:3869

**TubeA: tubeB的mol數比例取1：1**

**Backbone濃度: 3ng/ul**

**Insert濃度: 3ng/ul**

**Backbone: 1ul**

**Insert: 1.1 ul**

|  |  |
| --- | --- |
| **Reaction Component** | **A+B** |
| 10X Rapid Ligation Buffer, T4 DNA Ligase | 1μl |
| TubeA(backbone) | 4ul |
| TubeB(insert) | 4ul |
| T4 DNA Ligase  (3 Weiss units/μl) | 1μl |
| Total | **10μl** |

put in 25°C for 1hr

**Transformation & selection**

1.add 5(3.5)λ ligation sample into 100(70)λ competent cell

2. put on ice for 30 mins

3. Heat shock: 41℃ for 1min

4. put on ice for at least 10 mins

5. Add LB broth 900(630)ul to repair the cell wall

6. Culture in the 37℃ incubator for 1 hr，shaker 200 rpm

7. Centrifuge under 4krpm for 2 mins

8. Finish culturing in hood (AMP/CM petri dish)

9. put into 37 degrees incubator for at least 12 hrs

::Colony PCR

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 7.96 |
| 10x dream taq buffer | 1 |
| Primer VF2  (10uM) | 0.4 |
| Primer VR (10uM) | 0.4 |
| dNTP (2.5 mM) | 0.2 |
| Dream taq | 0.04 |
| Total | 10 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 2min |  |
| 95 | 30sec | X30 |
| 48(47.87) | 30sec |
| 72 | 2min(30sec) |
| 72 | 10min- |  |
| 20 | forever |  |

Plasmid extraction (”Presto Mini Plasmid Kit Quick Protocol”)

**DIGEST CHECK**

(1)

1.10XCutSmart buffer：1λ

2. ddH2O：6

3. template： 2

4. Not1HF： 0.5λ

5. Sac1HF： 0.5λ

Total： 10λ

**1648 2233**

(2)

1.10XNEB2.1 buffer：1λ

2. ddH2O：6

3. template： 2

4. HindIII： 0.5λ

5. Sac1HF： 0.5λ

Total： 10λ

**840 855 2186**