**pPIGBACK**

**Dream Taq**

5NSII

P0053 PBR322 ORI 5NSll

gcagaaaaaaaggatctcaacaggcaatcacgatgcgctg

P0052 5'-NSII-R

ataata GAATTCTCATTGCACACCCATCCATG

P0052: 62.18

P0053: 66.9

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| 7942 gDNA | 1 |
| Primer P0053(20uM) | 1 |
| Primer P0052(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 57.18 56 | 30sec |
| 72 | 1min30sec |
| 72 | 10min |  |
| 20 | - |  |

3NSll:

P0057 3'-NSII-F

gatgcg CTCGAGATCAACAAGCACACCATCAC

P0056 3NSII PBR322 ORI RC

Gggggcggagcctatggaaactgagtcgtgatgccaatgg

P0057: 55.26

P0056: 68.02

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| 7942 gDNA | 1 |
| Primer P0057(20uM) | 1 |
| Primer P0056(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 50.26 45 | 30sec |
| 72 | 1min30sec |
| 72 | 7min |  |
| 20 | - |  |

B0015

P0058 B0015-F

actagg GAATTCacggcc CCCGGGccaggcatcaaataaaacga

P0060 B0015 fusion amp-promoter+CDS-RC

AAACAAATAGGGGTTCCGCGtataaacgcagaaaggccca

P0058: 57.15

P0060: 61.82

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| Plate3,3F(dilute10X) | 1 |
| Primer P0058(20uM) | 1 |
| Primer P0060(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 52.15 45 | 30sec |
| 72 | 30sec |
| 72 | 7min |  |
| 20 | - |  |

amp-promoter+CDS:

P0059 B0015 fusion amp-promoter+CDS

TgggcctttctgcgtttataCGCGGAACCCCTATTTGTTT

P0061 AmpR promoter+CDS-RC

tggtgg CTCGAGTTACCAATGCTTAATCAGTG

P0059: 61.82

P0061: 49.9

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| PBR322 | 1 |
| Primer P0059(20uM) | 1 |
| Primer P0061(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 44.9 45 | 30sec |
| 72 | 1min30sec |
| 72 | 7min |  |
| 20 | - |  |

PBR322 ORI

P0055 3NSll PBR322 ORI

Ccattggcatcacgactcagtttccataggctccgccccc

P0054 PBR322 ORI 5NSII-AS

Cagcgcatcgtgattgcctgttgagatcctttttttctgc

P0055: 68.02

P0054: 66.9

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 18.75 |
| 10x Dream Taq buffer | 2.5 |
| PBR322 | 1 |
| Primer P0055(20uM) | 1 |
| Primer P0054(20uM) | 1 |
| dNTP (2.5mM) | 0.5 |
| Dream Taq 1U/ul | 0.25 |
| Total | 25 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |  |
| 95 | 5min |  |
| 95 | 45sec | X30 |
| 61.9 56 | 30sec |
| 72 | 50sec |
| 72 | 7min |  |
| 20 | - |  |

**KOD**

5NSII

P0053 PBR322 ORI 5NSll

gcagaaaaaaaggatctcaacaggcaatcacgatgcgctg

P0052 5'-NSII-R

ataata GAATTCTCATTGCACACCCATCCATG

P0052: 62.18

P0053: 66.9

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| 7942 gDNA | 2 |
| Primer P0052 (20uM) | 1 |
| Primer P0053 (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 |
| 52.18 48 | 30sec |
| 68 | 1min30sec |
| 68 | 10min |   |
| 20 | - |   |

3NSll:

P0057 3'-NSII-F

gatgcg CTCGAGATCAACAAGCACACCATCAC

P0056 3NSII PBR322 ORI RC

Gggggcggagcctatggaaactgagtcgtgatgccaatgg

P0057: 55.26

P0056: 68.02

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| 7942 gDNA | 2 |
| Primer P0057 (20uM) | 1 |
| Primer P0056 (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 |
| 45.26 48 | 30sec |
| 68 | 1min30sec |
| 68 | 10min |   |
| 20 | - |   |

B0015

P0058 B0015-F

actagg GAATTCacggcc CCCGGGccaggcatcaaataaaacga

P0060 B0015 fusion amp-promoter+CDS-RC

AAACAAATAGGGGTTCCGCGtataaacgcagaaaggccca

P0058: 57.15

P0060: 61.82

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| Plate3,3F(dilute 10X) | 2 |
| Primer P0052 (20uM) | 1 |
| Primer P0053 (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 |
| 47.15 48 | 30sec |
| 68 | 50sec |
| 68 | 10min |   |
| 20 | - |   |

amp-promoter+CDS:

P0059 B0015 fusion amp-promoter+CDS

TgggcctttctgcgtttataCGCGGAACCCCTATTTGTTT

P0061 AmpR promoter+CDS-RC

tggtgg CTCGAGTTACCAATGCTTAATCAGTG

P0059: 61.82

P0061: 49.9

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| PBR322 | 2 |
| Primer P0059 (20uM) | 1 |
| Primer P0061 (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 |
| 39.9 | 30sec |
| 68 | 1min30sec |
| 68 | 10min |   |
| 20 | - |   |

PBR322 ORI

P0055 3NSll PBR322 ORI

Ccattggcatcacgactcagtttccataggctccgccccc

P0054 PBR322 ORI 5NSII-AS

Cagcgcatcgtgattgcctgttgagatcctttttttctgc

P0055: 68.02

P0054: 66.9

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 14 |
| 10x KOD PLUS buffer | 2.5 |
| PBR322 | 2 |
| Primer P0055 (20uM) | 1 |
| Primer P0054 (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 |
| 56.9 48 | 30sec |
| 68 | 1min |
| 68 | 10min |   |
| 20 | - |   |

0830

All dream taq (electrophoresis :5NS2(于), 3NS2(于), ORI, B0015(Plate3F dilute 10x), AMP)

**0831**

**morning**

Dream taq: 5NS2, ORI, B0015

(electrophoresis : 5NS2, ORI-1(PBR322-1), ORI-2(PBR322-2), B0015-1(NrtA033), B0015-2(Plate3F dilute 10x)

KOD: 3NS2, AMP(electrophoresis: AMP-1(PBR322-1), AMP-2(PBR322-2), 3NS2-1(于), 3NS2-2(Jo))

**noon**

KOD: 5NS2, 3NS2, ORI, B0015 (5NS2, ORI Tm: 53/ 3NS2, B0015: 47 )

Dream taq: ORI

**Fusion PCR**

**(1) fusion: 5NS2, 3NS2, ORI**

P0052 5'-NSII-R: ataata GAATTCTCATTGCACACCCATCCATG

P0057 3'-NSII-F

gatgcg CTCGAGATCAACAAGCACACCATCAC

All cut gel purified and dissolved in 15 ul ddH2O

Load 1 ul for concentration check

**TubeA**

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 61 |
| 10x KOD PLUS buffer | 10 |
| 5NSII | 2 |
| 3NS2 | 2 |
| ORI | 1 |
| Primer P0052 (20uM) | 4 |
| Primer P0057 (20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 4 |
| KOD PLUS polymerasae 1U/ul | 2 |
| Total | 100 |

3NSll ORI 5NSII concentration 32ng/ul

Mole 1:1:1 🡪 volume: 3NSll: ORI:5NSII= 1:0.5:1

P0052 Tm: 68.34

P0057 Tm: 77.12

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |   |
| 94 | 2min |   |
| 94 | 20sec | X5 |
| 51  | 30sec |
| 68 | 3min30sec |
| Pause and add 2ul Pimer P0052, 2ul Primer P0057 |
| 94 | 20sec | X30 |
| 58.34 | 30sec |
| 68 | 3min30sec |
| 68 | 10min |   |
| 20 | - |  |

**Fusion PCR**

**(2) fusion: AMP, B0015**

P0058 B0015-F: actagg GAATTCacggcc CCCGGGccaggcatcaaataaaacga

P0061AmpR promoter+CDS –R

tggtgg CTCGAGTTACCAATGCTTAATCAGTG

All cut gel purified and dissolved in 15 ul ddH2O

Load 1 ul for concentration check

**TubeB**

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 63.8 |
| 10x KOD PLUS buffer | 10 |
| AMP | 2 |
| B0015 | 0.4 |
| Primer P0058 (20uM) | 4 |
| Primer P0061 (20uM) | 4 |
| dNTP (2mM) | 10 |
| 25mM MgSO4 | 4 |
| KOD PLUS polymerasae 1U/ul | 2 |
| Total | 100 |

AMP B0015 concentration: 32ng/ul

Mole 1:1->volume : AMP:B0015= 1:0.1

P0058 Tm: 85.76

P0061 Tm: 72.36

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |   |
| 94 | 2min |   |
| 94 | 20sec | X5 |
| 51  | 30sec |
| 68 | 1min30sec |
| Pause and add 2ul Pimer P0058, 2ul Primer P0061 |
| 94 | 20sec | X30 |
| 62.36 (58.34) | 30sec |
| 68 | 1min30sec |
| 68 | 10min |  |
| 20 | - |  |

**Digestion**

10Xcut smart buffer：2λ

template： 14λ

EcoR1-HF: 2λ

Xho1：2λ

Total： 20λ

put into 37 degrees incubator for at least 1 hr

**PCR clean up (ddH2O elute 15ul)**

**Load 1 ul for concentration check**

**Ligation**

**TubeA: tubeB mole: 1：1**

TubeA(BACKBONE): 30 ng/ul

TubeB(insert): 50 ng/ul

**TubeA:1 ul**

**TubeB: 0.24ul**

|  |  |
| --- | --- |
| **Reaction Component** | **A+B** |
| 10X Rapid Ligation Buffer | 1μl |
| TubeA(backbone) | 6.4 ul |
| TubeB(insert) | 1.6μl |
| T4 DNA Ligase(3 Weiss units/μl) | 1μl |
| Total | **10μl** |

put 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**

P0053 PBR322 ORI 5NSll-S

gcagaaaaaaaggatctcaacaggcaatcacgatgcgctg

P0060 B0015 fusion amp-promoter+CDS-AS

AAACAAATAGGGGTTCCGCGtataaacgcagaaaggccca

P0053: 67.6

P0060:67.6

|  |  |
| --- | --- |
| Reaction Component | Volume (ul) |
| ddH2O | 7.96 |
| 10x dream taq buffer | 1 |
| P0053 | 0.4 |
| P0060 | 0.4 |
| dNTP (2.5 mM) | 0.2 |
| Dream taq | 0.04 |
| Total | 10 |

|  |  |  |
| --- | --- | --- |
| Reaction Temperature | Time |   |
| 95 | 2min |   |
| 95 | 30sec | X30 |
| 62.6 | 30sec |
| 72 | 1min30sec |
| 72 | 10min- |  |
| 20 | forever |  |

**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**

**control:**

**NrtA HindIII, SacI 1285 2316**

**NrtA NotI, SacI 907, 1139, 1555**