**Generation of a PB1 defective interfering virus**

Preparation of polI-DI477 expression construct

- PCR amplification of H7\_PB1-DI477 fragments 1-3

- 5’ cloning site: BsmBI

- 3’ cloning site: BsmBI

- forward primer of fragment 1: tagcacgtctcgggggagcaaaagcaggcaaaccat

- reverse primer of fragment 1: ttgcactggcaagaaaagtaaagtcggattg

- forward primer of fragment 2: atccgactttacttttcttgccagtgcaaaatgctataagtaccactt

- reverse primer of fragment 2: gttgcagcacttctggtaattggattgagttggggtgctccagtctc

- forward primer of fragment 3: accccaactcaatccaattaccagaagtgctgcaacctattcgaaaagtt

- reverse primer of fragment 3: tagcacgtctcgtattagtagaaacaaggcattttttc

- template: pHW2000-H7\_PB1

- enzyme: KAPA HiFi HotStart PCR Kit

PCR reaction:

|  |  |
| --- | --- |
| Template | 0.5 uL |
| 5x KAPA HiFi buffer | 5 uL |
| KAPA dNTP Mix (10mM ) | 0.75 uL |
| Forward primer (10uM) | 0.5 uL |
| Reverse primer (10uM) | 0.5 uL |
| KAPA HiFi Hotstart DNA polymerase (1U/uL) | 0.5 uL |
| ddH2O | 17.25 uL |
| Total | 25 uL |

- PCR condition:

|  |  |  |  |
| --- | --- | --- | --- |
| Initial denaturation | 95ºC | 3 mins | 1 cycle |
| Denaturation | 98ºC | 20 secs | 25 cycles |
| Annealing | 60ºC | 15 secs |
| Extension | 72ºC | 20 secs |
| Final extension | 72ºC | 3 mins | 1 cycle |

- PCR product of fragments 1-3 are gel cleaned for overlapping PCR

- overlapping PCR reaction:

- PCR amplification of H7\_PB1-DI477

- 5’ cloning site: BsmBI

- 3’ cloning site: BsmBI

- forward primer: tagcacgtctcgggggagcaaaagcaggcaaaccat

- reverse primer: tagcacgtctcgtattagtagaaacaaggcattttttc

- template: gel cleaned product of fragments 1-3

- enzyme: KAPA HiFi HotStart PCR Kit

|  |  |
| --- | --- |
| Template | 1:1:1 molar ratio of fragments 1-3 (~100ng) |
| 5x KAPA HiFi buffer | 5 uL |
| KAPA dNTP Mix (10mM ) | 0.75 uL |
| Forward primer (10uM) | 0.5 uL |
| Reverse primer (10uM) | 0.5 uL |
| KAPA HiFi Hotstart DNA polymerase (1U/uL) | 0.5 uL |
| ddH2O | Top up to 25 uL |
| Total | 25 uL |

- PCR condition:

|  |  |  |  |
| --- | --- | --- | --- |
| Initial denaturation | 95ºC | 3 mins | 1 cycle |
| Denaturation | 98ºC | 20 secs | 25 cycles |
| Annealing | 60ºC | 15 secs |
| Extension | 72ºC | 30 secs |
| Final extension | 72ºC | 3 mins | 1 cycle |

- PCR product is gel cleaned for restriction enzyme digestion

- restriction enzyme digestion condition

|  |  |
| --- | --- |
| DNA | 1 ug |
| NEBuffer 3.1 | 5 uL |
| BsmBI | 1 uL |
| ddH2O | Top up to 50 uL |

- incubate at 55ºC overnight

- ligation of PCR product and pLV-EF1a-hygro and transformation to Stbl3 competent cells

- midiprep of plasmid DNA

Reverse genetics

- co-culture of MDCK-2P and 293FT-2P in 6-well plate

- one day after seeding, confluence reach 70% for transfection

|  |  |  |  |
| --- | --- | --- | --- |
| pHW2000-H7\_PB2 | polI-PB1-DI477 | pHW2000-H7\_PA | pHW2000-H7\_NP |
| polI-WSN\_HA | polI-WSN\_NA | polI-WSN\_M | polI-WSN\_NS |

- total of 2.5ug of DNA and 7.5uL of lipofectamine 2000 were mixed in Opti-MEM and incubated for 20 mins

- replace the medium with plain MEM 6h after transfection

- add 1:5000 TPCK-trypsin on the next day

- 72h post transfection, harvest the medium and centrifuge at 4000rpm for 5 mins at 4C

- passage the virus-containing supernatant to fresh MDCK-2P on a 6-well plate

VIral RNA extraction

- lyse 140uL of viral supernatant with buffer AvL of QIamp Viral RNA mini kit

- perform the RNA extraction process according to manufacturer’s protocol

RT-PCR of H7-WSN-DI477

- template: H7-WSN-DI477 viral RNA

- primer: agcaaaagcagg

- enzyme: PrimeScript RT reaction kit

- RT-PCR condition:

gDNA removal

|  |  |
| --- | --- |
| Viral RNA | 7 uL |
| 5x gDNA eraser buffer | 2 uL |
| gDNA eraser | 1 uL |
| Total | 10 uL |

- 42ºC for 2 mins

RT-PCR

|  |  |
| --- | --- |
| Reaction mixture from gDNA removal | 10 uL |
| 5x PrimeScript buffer 2 | 4 uL |
| primer | 0.5 uL |
| PrimeScript RT Enzyme Mix I | 1 uL |
| ddH2O | 4.5 uL |
| Total | 20 uL |

PCR amplification of H7-WSN-DI477 cDNA

- PB2 forward primer: gatttgatgtcacagtctcgcactc

- PB2 reverse primer: cgaatccttttggtcgctgtctg

- PB1 forward primer: atggatgtcaatccgactttacttttc

- PB1 reverse primer: ctatttttgccgtctgagctcttcaatg

- PA forward primer: tgcgacaatgcttcaatccaatg

- PA reverse primer: tatcttagtgcatgtgtgaggaaggag

- template: H7-WSN-DI477 cDNA

- enzyme: DreamTaq DNA polymerase

PCR reaction:

|  |  |
| --- | --- |
| Template | 0.5 uL |
| 10x DreamTaq buffer | 2.5 uL |
| dNTP Mix (10mM ) | 0.5 uL |
| Forward primer (10uM) | 0.5 uL |
| Reverse primer (10uM) | 0.5 uL |
| DreamTaq DNA polymerase | 0.125 uL |
| ddH2O | 20.375 uL |
| Total | 25 uL |

- PCR condition:

|  |  |  |  |
| --- | --- | --- | --- |
| Initial denaturation | 95ºC | 3 mins | 1 cycle |
| Denaturation | 95ºC | 30 secs | 30 cycles |
| Annealing | 60ºC | 30 secs |
| Extension | 72ºC | 1 mins and 10 secs |
| Final extension | 72ºC | 3 mins | 1 cycle |

TA cloning of PB2 DI bands

- excise and gel clean the DNA gel containing the PB2 DI bands

- ligate the gel clean product with pGEM-T easy vector at 16ºC overnight and transform into DH5a

- add 10uL 1M IPTG and 50uL 20mg/mL X-gal to the agar plate for blue-white selection

- select white colonies for Sanger sequencing