

Cloning of orf6 & orf9b plasmids

(1) PCR amplification of cloning orf6&9b

28/3/2022

- Orf6 WT
- Orf6 D61L
- Orf9b WT
- Orf9b Omi fragment 2
- Orf9b Delta fragment 1
- Orf9b Delta fragment 2

	SARS2-orf6-WT (2X)
5X GXL Buffer	10 µL
Primer set	1 µL NheI-Orf6-WT-F (10µM)
	1 µL BamHI-Orf6-WT-R (10µM)
2.5 mM dNTP	4 µL
Template	1 µL (10ng/µL)
GXL DNA Polymerase	1 µL
DDH ₂ O	32 µL
Total	50 µL

	SARS2-orf6-D61L (2X)
5X GXL Buffer	10 µL
Primer set	1 µL NheI-Orf6-WT-F (10µM)
	1 µL BamHI-Orf6-D61L-R (10µM)
2.5 mM dNTP	4 µL
Template	1 µL (10ng/µL)
GXL DNA Polymerase	1 µL
DDH ₂ O	32 µL
Total	1 50µL

	SARS2-orf9b-WT (2X)
5X GXL Buffer	10 µL

Primer set	1 μ L NheI-Orf9b-WT-F (10 μ M)
	1 μ L BamHI-Orf9b-WT-R (10 μ M)
2.5 mM dNTP	4 μ L
Template	1 μ L (10ng/ μ L)
GXL DNA Polymerase	1 μ L
DDH₂O	32 μ L
Total	50 μ L

	SARS2-orf9b-Delta (1st fragment) (2X)
5X GXL Buffer	10 μ L
Primer set	1 μ L NheI-Orf9b-WT-F (10 μ M)
	1 μ L Orf9b-Delta-R (10 μ M)
2.5 mM dNTP	4 μ L
Template	1 μ L (10ng/ μ L)
GXL DNA Polymerase	1 μ L
DDH₂O	32 μ L
Total	50 μ L

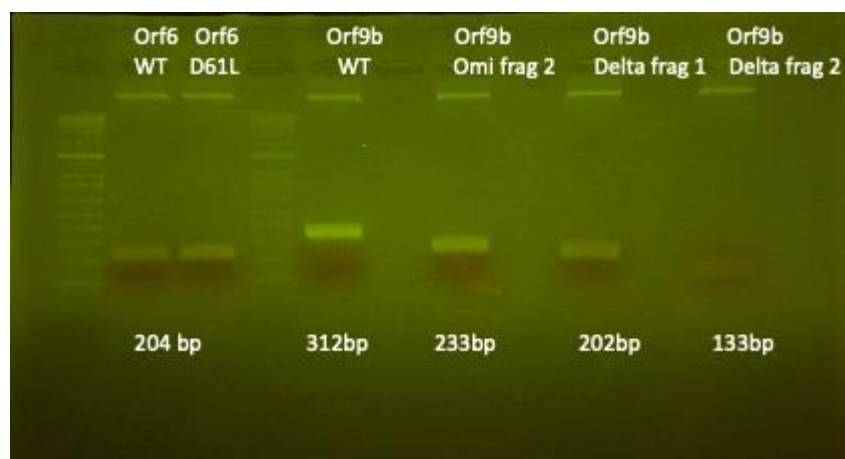
	SARS2-orf9b-Delta (2nd fragment) (2X)
5X GXL Buffer	10 μ L
Primer set	1 μ L Orf9b-Delta-F (10 μ M)
	1 μ L BamHI-Orf9b-WT-R (10 μ M)
2.5 mM dNTP	4 μ L
Template	1 μ L (10ng/ μ L)
GXL DNA Polymerase	1 μ L
DDH₂O	32 μ L
Total	50 μ L

	SARS2-orf9b-Omicron (2nd fragment) (2X)
5X GXL Buffer	10 μ L
Primer set	1 μ L BsmBI-Orf9b-Omi-2-F (10 μ M)
	1 μ L BsmB1-Orf9b-Omi-2-R (10 μ M)
2.5 mM dNTP	4 μ L
Template	1 μ L (10ng/ μ L)
GXL DNA Polymerase	1 μ L
DDH₂O	32 μ L

Total	50 μ L
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1. PCR setting

		Temperature	Time
Initial denature		98 °C	3mins
Cycling (20 cycles)	Denature	98 °C	20s
	Annealing	60 °C	15s
	Extension	68 °C	30 sec
Final Extension		68 °C	5mins
Store		10 °C	∞



Agarose Gel electrophoresis :

2. Prepare 50mL 2% agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
3. Cool the gel solution a little bit, then add 2.5 μ L (1:20000) SmartGlow to the gel solution and mix well
4. Pour the gel to the gel rack and wait for solidification.
5. Transfer the gel to the gel tank soaked with 1X TAE buffer
6. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.

7. Run the gel at 120V (constant voltage) for 24mins.
8. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendorf (better to keep gel slice below 0.2 g)

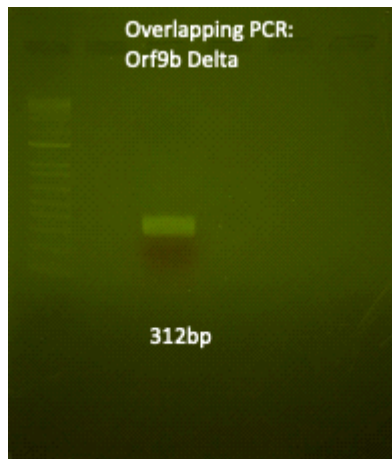
Gel Purification – GeneJET Gel Extraction Kit (#K0691, #K0692)

9. Add 1:1 volume (μL) to gel weight (mg) of Binding Buffer to the eppendorf containing the gel (e.g add 100 μL to a 100mg gel piece)
10. Heat at 50°C on the heat block until the gel is fully dissolved (**Caution: A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 μL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.)
 - Add 1:1 volume (μL) to gel weight (mg) of 100% isopropanol to the solubilized gel solution if DNA fragment is ≤ 500 bp
 - Add 1:1 volume (μL) to gel weight (mg) of ddH₂O to the solubilized gel solution if DNA fragment is >10 kb
11. Transfer up to 800 μL of the solubilized gel solution (from step 3 or 4) to the GeneJET purification column
12. Centrifuge at 13,000 g ($>12,000$ g) for 1min
13. Discard the flow through
14. Repeat Step 3-5 until all dissolved solution is through flow the column
15. Add 700 μL Wash buffer containing Ethanol to the column
16. Centrifuge at $20,817 \times g$ (max speed) for 1min
17. Discard the flow through
18. Centrifuge at $20,817 \times g$ (max speed) for an additional 2mins to completely remove residual wash buffer
19. Replace the disposable tube with a Recovery Tube, add **30 μL Elution Buffer** to the center of the column
20. Centrifuge at $20,817 \times g$ (max speed) for 2mins

Overlapping PCR of Orf9b Delta

29/3/2022

	SARS2-orf9b-Delta (Overall) (2X)
5X GXL Buffer	10 µL
Primer set	1 µL NheI-Orf9b-WT-F (10µM)
	1 µL BamHI-Orf9b-WT-R (10µM)
2.5 mM dNTP	4 µL
Template	1 uL PCR product (1 st frag) 1 uL PCR product (2 nd frag)
GXL DNA Polymerase	1 µL
DDH ₂ O	31 µL
Total	50 µL



(2) Restriction enzyme digestion

29/3/2022

- Orf6 WT
- Orf6 D61L (Omicron)
- Orf9b WT
- Orf9b T60A (Delta)

	Amount
Gel purified PCR product (Insert)	30 µL
Restriction Digestion Buffer (CutSmart)	5 µL

NheI-HF	1 µL
BamHI-HF	1 uL
ddH2O	13 uL
Total	50 µL

	Amount
pcDNA3.1-IRES-GFP (1ug) (Vector)	1 µL
Restriction Digestion Buffer (CutSmart)	5 µL
NheI-HF	1 µL
BamHI-HF	1 uL
ddH2O	43 uL
Total	50 µL

Incubate at 37°C overnight

Restriction enzyme digestion (Orf9b Omicron)

30/3/2022

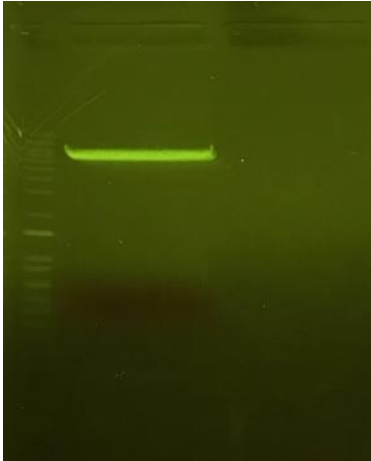
	Amount
Gel purified PCR product (Insert)	30 µL
Restriction Digestion Buffer (NEB Buffer 3.1)	5 µL
BsmBI	1 uL
ddH2O	14 uL
Total	50 µL

Incubate at 55°C for 1 hr

Agarose Gel electrophoresis (Enzyme digested Vector)

21. Prepare 50mL **0.5%** agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
22. Cool the gel solution a little bit, then add 2.5µL (1:20000) SmartGlow to the gel solution and mix well
23. Pour the gel to the gel rack and wait for solidification.

24. Transfer the gel to the gel tank soaked with 1X TAE buffer
25. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
26. Run the gel at 120V (constant voltage) for 24mins.
27. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendrof (better to keep gel slice below 0.2 g)



Gel Purification (GeneJET Gel Extraction and DNA Cleanup Micro Kit)

28. Add **200 μ L** of **Extraction Buffer** to the eppendrof containing the
29. Heat at 50°C on the heat block for 10 minutes or until the gel slice is completely dissolved
30. Add **200 μ L** of **absolute ethanol (96-100%)** and mix by pipetting
31. Transfer the mixture to the DNA Purification Micro Column (white) preassembled with a collection tube.
32. Centrifuge the column at 20,817 \times g (max speed) for 1min
33. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
34. Add **200 μ L** of **Prewash Buffer** (supplemented with ethanol) to the column
35. Centrifuge the column at 20,817 \times g (max speed) for 1min

36. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
37. Add **700 µL of Wash Buffer** (supplemented with ethanol) to the column
38. Centrifuge the column at $20,817 \times g$ (max speed) for 1min
39. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
40. Add **700 µL of Wash Buffer** (supplemented with ethanol) to the column
41. Centrifuge the column at $20,817 \times g$ (max speed) for 1min
42. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
43. Centrifuge the column at $20,817 \times g$ (max speed) for addition 1min to completely remove residual Wash Buffer
44. Transfer the DNA Purification Micro Column into a clean 1.5 mL eppendrof
45. Add **20 µL of warmed ddH₂O** to the DNA Purification Micro Column.
46. Centrifuge at $20,817 \times g$ (max speed) for 1min to elute DNA

(3) Ligation

30/3/2022

Others:

	Amount
10X ligation buffer	1µL
Insert	0.3 µL
Vector (pcDNA3.1-IRES-GFP)	1µL
T4 DNA ligase	0.5µL
ddH₂O	7.2 uL
Total	10µL

Orf9b Omicron:

	Amount
10X ligation buffer	1µL

Fragment 1	0.5 μ L
Fragment 2	0.5 μ L
Vector (pcDNA3.1-IRES-GFP)	1 μ L
T4 DNA ligase	0.5 μ L
ddH₂O	6.5 μ L
Total	10μL

Room temperature overnight

(4) Transformation

31/3/2022

1. Thaw the competent cell (DH5 α *E.coli*) on ice and also place the ligation product on ice for at least 1min
2. Add 50 μ L competent cell to the 10 μ L ligation product, flap the tubes to brief mixing (Do not vortex!!), and place back on ice for 20mins
3. Heat shock the competent cell – ligation product mix on 42°C water bath or heat block for 1 min
4. Place back on ice immediately for 5mins
5. Add 500 μ L plain LB broth to the tubes and incubate at 37°C incubator with shaking for 1hr (Amp resistant)
6. Centrifuge the bacteria at max speed for 1min
7. Remove the supernatant to ~50 μ L LB broth left in the tubes
8. Re-suspend the bacteria with this ~50 μ L LB and spread the bacteria to LB (Amp) plate
9. Incubate at 37°C incubator for 12-16 hours overnight

Pick colonies

1. Aliquot 500 μ L LB(Amp) to a 1.5mL eppendrof
2. Pick 4 colonies of each reaction to the corresponding 500 μ L growth medium
3. Incubate at 37°C incubator for ~6 hours with shaking

PCR Screening

1/4/2022

PCR Reaction:

	Orf6 WT
Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L CMV-F
	0.8 μ L BamHI-Orf6-WT-R
dNTP	0.4 μ L
Template	colony
Dream Taq	0.1 μ L
DDH₂O	14.9 μ L
Total	20 μ L

Target size: 332bp

	Orf6 Omi
Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L CMV-F
	0.8 μ L BamHI-Orf6-D61L-R
dNTP	0.4 μ L
Template	colony
Dream Taq	0.1 μ L
DDH₂O	14.9 μ L
Total	20 μ L

Target size: 332bp

	Orf9b WT
Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L CMV-F
	0.8 μ L BamHI-Orf9b-WT-R
dNTP	0.4 μ L
Template	colony
Dream Taq	0.1 μ L
DDH₂O	14.9 μ L

Total	20 µL
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Target size: 443bp

	Orf9b Delta
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL CMV-F
	0.8 µL BamHI-Orf9b-WT-R
dNTP	0.4 µL
Template	colony
Dream Taq	0.1 µL
DDH₂O	14.9 µL
Total	20 µL

Target size: 443bp

	Orf9b Omi
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL CMV-F
	0.8 µL BsmB1-Orf9b-Omi-2-R
dNTP	0.4 µL
Template	colony
Dream Taq	0.1 µL
DDH₂O	14.9 µL
Total	20 µL

Target size: 439bp

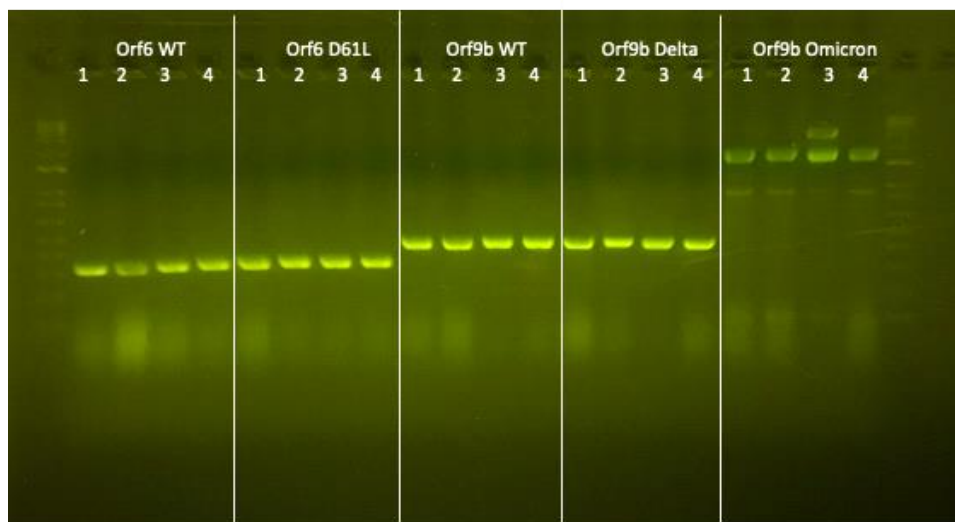
PCR setting

		Temperature	Time
Initial denature		95 °C	3mins
Cycling (30 cycles)	Denature	95 °C	30s
	Annealing	55 °C	30s
	Extensiton	72 °C	1min (2kb/1min, then 1kb/min)
Final Extension		72 °C	5mins

Store		4 °C	∞
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Agarose Gel electrophoresis :

1. Prepare 50mL **2%** agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
2. Cool the gel solution a little bit, then add 2.5μL (1:20000) SmartGlow to the gel solution and mix well
3. Pour the gel to the gel rack and wait for solidification.
4. Transfer the gel to the gel tank soaked with 1X TAE buffer
5. Add 10μL 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
6. Run the gel at 120V (constant voltage) for 24mins.
7. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendrof (better to keep gel slice below 0.2 g)



Orf9b Omicron --> **WRONG** size

Others correct !!!

For orf9b Omi --> Order gene synthesis

Orf9b Omi (Gene Synthesis)

24/5/2022

(1) Redissolve m9b (Orf9b Omicron) plasmid

Add 50uL TE buffer to redissolve the plasmid
Warm in 50 °C heat block for 3 min

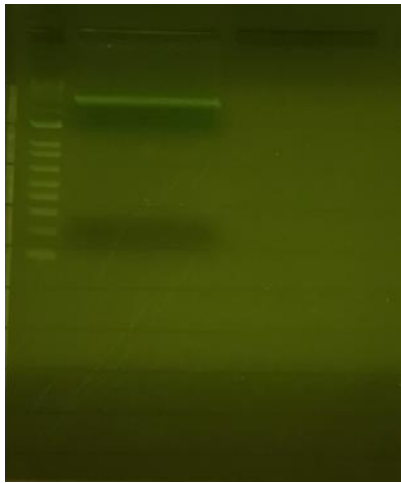
(2) Enzyme digestion (Orf9b Omicron)

	Amount
Redissolved m9b plasmid (2ug) (Insert)	10 µL
Restriction Digestion Buffer (CutSmart)	5 µL
NheI-HF	1 µL
BamHI-HF	1 uL
ddH2O	33 uL
Total	50 µL

Incubate at 37°C for 3 hours

Agarose Gel electrophoresis

1. Prepare 25mL 0.5% agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
2. Cool the gel solution a little bit, then add 1.25µL (1:20000) SmartGlow to the gel solution and mix well
3. Pour the gel to the gel rack and wait for solidification.
4. Transfer the gel to the gel tank soaked with 1X TAE buffer
5. Add 10µL 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
6. Run the gel at 120V (constant voltage) for 24mins.
7. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendrof (better to keep gel slice below 0.2 g)



--> can't see the band (~303bp) --> PCR amplification instead

25/5/2022

(1) PCR Amplification

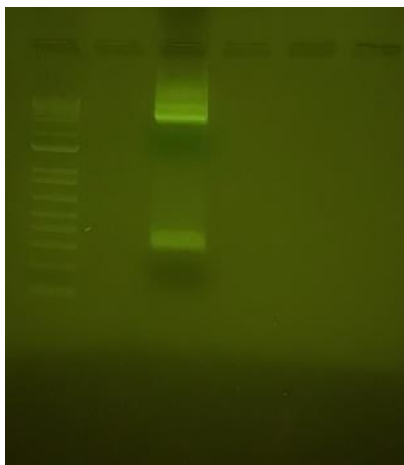
	SARS2-orf9b-Omicron (2 nd fragment) (2X)
5X GXL Buffer	5 µL
Primer set	0.5 µL NheI-Orf9b-WT-F (10µM)
	0.5 µL BamHI-Orf9b-WT-R (10µM)
2.5 mM dNTP	4 µL
Template	1 µL m9b plasmid
GXL DNA Polymerase	0.5 µL
DDH ₂ O	14.5 µL
Total	25 µL

Target size: 312bp

		Temperature	Time
Initial denature		98 °C	3mins
Cycling (20 cycles)	Denature	98 °C	20s
	Annealing	60 °C	15s
	Extension	68 °C	30 sec
Final Extension		68 °C	5mins
Store		10 °C	∞

Agarose Gel electrophoresis :

8. Prepare 25mL **2%** agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
9. Cool the gel solution a little bit, then add 1.25 μ L (1:20000) SmartGlow to the gel solution and mix well
10. Pour the gel to the gel rack and wait for solidification.
11. Transfer the gel to the gel tank soaked with 1X TAE buffer
12. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
13. Run the gel at 120V (constant voltage) for 24mins.
14. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendrof (better to keep gel slice below 0.2 g)



Gel Purification – GeneJET Gel Extraction Kit (#K0691, #K0692)

1. Add 1:1 volume (μ L) to gel weight (mg) of Binding Buffer to the eppendrof containing the gel (e.g add 100 μ L to a 100mg gel piece)
2. Heat at 50°C on the heat block until the gel is fully dissolved (**Caution: A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 μ L of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.)
 - **Add 1:1 volume (μ L) to gel weight (mg) of 100% isopropanol to the solubilized gel solution if DNA fragment is \leq 500 bp**
 - Add 1:1 volume (μ L) to gel weight (mg) of ddH₂O to the solubilized gel solution if DNA fragment is >10 kb

3. Transfer up to 800 μL of the solubilized gel solution (from step 3 or 4) to the GeneJET purification column
4. Centrifuge at 13,000 g ($>12,000$ g) for 1min
5. Discard the flow through
6. Repeat Step 3-5 until all dissolved solution is through flow the column
7. Add 700 μL Wash buffer containing Ethanol to the column
8. Centrifuge at $20,817 \times g$ (max speed) for 1min
9. Discard the flow through
10. Centrifuge at $20,817 \times g$ (max speed) for an additional 2mins to completely remove residual wash buffer
11. Replace the disposable tube with a Recovery Tube, add **30 μL Elution Buffer** to the center of the column
12. Centrifuge at $20,817 \times g$ (max speed) for 2mins

(2) Enzyme digestion (Orf9b Omicron)

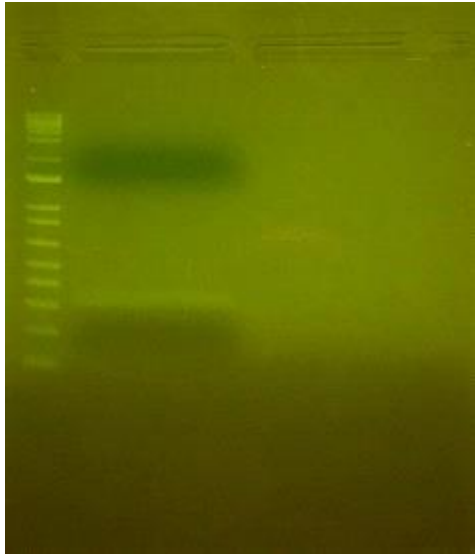
	Amount
Gel purified PCR product (Insert)	30 μL
Restriction Digestion Buffer (CutSmart)	5 μL
NheI-HF	1 μL
BamHI-HF	1 μL
ddH₂O	13 μL
Total	50 μL

Incubate at 37°C for 3 hours

Agarose Gel electrophoresis

13. Prepare 25mL **2%** agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
14. Cool the gel solution a little bit, then add 1.25 μL (1:20000) SmartGlow to the gel solution and mix well
15. Pour the gel to the gel rack and wait for solidification.
16. Transfer the gel to the gel tank soaked with 1X TAE buffer

17. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
18. Run the gel at 120V (constant voltage) for 24mins.
19. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendorf (better to keep gel slice below 0.2 g)



Gel Purification – GeneJET Gel Extraction Kit (#K0691, #K0692)

20. Add 1:1 volume (μ L) to gel weight (mg) of Binding Buffer to the eppendorf containing the gel (e.g add 100 μ L to a 100mg gel piece)
21. Heat at 50°C on the heat block until the gel is fully dissolved (**Caution: A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 μ L of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.)
 - Add 1:1 volume (μ L) to gel weight (mg) of 100% isopropanol to the solubilized gel solution if DNA fragment is ≤ 500 bp
 - Add 1:1 volume (μ L) to gel weight (mg) of ddH₂O to the solubilized gel solution if DNA fragment is >10 kb
22. Transfer up to 800 μ L of the solubilized gel solution (from step 3 or 4) to the GeneJET purification column
23. Centrifuge at 13,000 g ($>12,000$ g) for 1min
24. Discard the flow through
25. Repeat Step 3-5 until all dissolved solution is through flow the column
26. Add 700 μ L Wash buffer containing Ethanol to the column

27. Centrifuge at $20,817 \times g$ (max speed) for 1min
28. Discard the flow through
29. Centrifuge at $20,817 \times g$ (max speed) for an additional 2mins to completely remove residual wash buffer
30. Replace the disposable tube with a Recovery Tube, add **30 μ L Elution Buffer** to the center of the column
31. Centrifuge at $20,817 \times g$ (max speed) for 2mins

(3) Ligation (Orf9b Omi)

	Amount
10X ligation buffer	1 μ L
Insert	0.3 μ L
Vector (pcDNA3.1-IRES-GFP)	1 μ L
T4 DNA ligase	0.5 μ L
ddH ₂ O	7.2 uL
Total	10μL

	Amount
10X ligation buffer	1 μ L
Insert	0.5 μ L
Vector (pcDNA3.1-IRES-GFP)	1 μ L
T4 DNA ligase	0.5 μ L
ddH ₂ O	7 uL
Total	10μL

Incubate at room temperature overnight

(4) Transformation (Orf9b Omi)

26/5/2022

Thaw the competent cell (DH5 α *E.coli*) on ice and also place the ligation product on ice for at least 1min

Add **4uL ligation product** to competent cell to the, flap the tubes to brief mixing (Do not vortex!!), and place back on ice for 20mins

Heat shock the competent cell – ligation product mix on 42°C heat block for 1 min

Place back on ice immediately for 5mins

Add 500µL plain LB broth to the tubes and incubate at 37°C incubator with shaking for 1hr

Centrifuge the bacteria at max speed for 1min

Remove the supernatant to ~100µL LB broth left in the tubes

Re-suspend the bacteria with this ~100µL LB and spread the bacteria to LB **(Amp)** plate

Incubate at 37°C incubator for 12-16 hours

(5) Pick colonies & PCR Screening (Orf9b Omi)

Setup for PCR reactions

Dream Taq Green Buffer	2 µL
Primer set	0.8 µL CMV F
	0.8 µL BamHI-Orf9b-WT-R
dNTP	0.4 µL
Template	1 µL
Dream Taq	0.1 µL
DDH₂O	15.9 µL
Total	20 µL

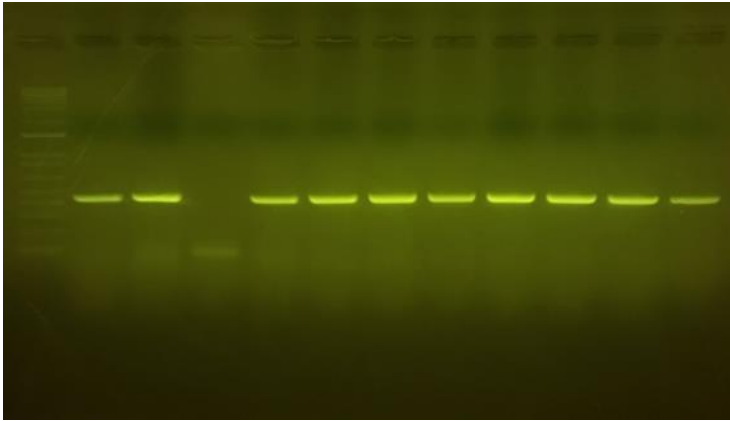
Target size: 439bp

	Temperature	Time
Initial denature	95 °C	10mins
Denature	95 °C	30s
Annealing	55 °C	30s
Extension	72 °C	1min (2kb/1min, then 1kb/min)
Final Extension	72 °C	5mins
Store	4 °C	∞

Agarose Gel electrophoresis

32. Prepare 50mL **2%** agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
33. Cool the gel solution a little bit, then add 2.5µL (1:20000) SmartGlow to the gel solution and mix well

34. Pour the gel to the gel rack and wait for solidification.
35. Transfer the gel to the gel tank soaked with 1X TAE buffer
36. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
37. Run the gel at 120V (constant voltage) for 24mins.
38. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendorf (better to keep gel slice below 0.2 g)



Expect colony 3, others correct !!!

Midi-prep & Send sequencing

Transform MV-V plasmid to DH5 α

7/4/2022

1. Thaw the competent cell (DH5 α *E.coli*) on ice and also place the ligation product on ice for at least 1min
2. Add 50 μ L competent cell to the 1 μ L plasmid , flap the tubes to brief mixing (Do not vortex!!), and place back on ice for 20mins
3. Heat shock the competent cell – ligation product mix on 42°C water bath or heat block for 1 min
4. Place back on ice immediately for 5mins
5. Add 500 μ L plain LB broth to the tubes and incubate at 37°C incubator with shaking for 1hr (Amp resistant)
6. Centrifuge the bacteria at max speed for 1min
7. Remove the supernatant to ~50 μ L LB broth left in the tubes
8. Re-suspend the bacteria with this ~50 μ L LB and spread the bacteria to LB (Amp) plate
9. Incubate at 37°C incubator for 12-16 hours overnight

Pick colonies

8/4/2022

10. Aliquot 500 μ L LB(Amp) to a 1.5mL eppendorf
11. Pick 1 colony of each reaction to the corresponding 500 μ L growth medium
12. Incubate at 37°C incubator for ~6 hours with shaking

Subculture for Midi-prep

10/4/2022

- Orf6 WT colony 1
- Orf6 D61L colony 3
- MV-V colony 1

1. Add 50 μ L bacteria to 50mL LB(Amp)
2. Incubate at 37°C, 250rpm for 12-16 hours

24/4/2022

- Orf9b WT colony 1
- Orf9b Delta colony 3

1. Add 50uL bacteria to 50mL LB(Amp)
2. Incubate at 37°C, 250rpm for 12-16 hours

31/5/2022

- Orf9b Omi colony 2

1. Add 50uL bacteria to 50mL LB(Amp)
2. Incubate at 37°C, 250rpm for 12-16 hours

Midi-prep

11/4/2022

- Orf6 WT colony 1
- Orf6 D61L colony 3
- MV-V colony 1

25/4/2022

- Orf9b WT colony 1
- Orf9b Delta colony 3

1/6/2022

- Orf9b Omi colony 2

1. Pour 50mL growth overnight culture in a 50mL centrifuge tube
2. Centrifuge at 6000x g for 15mins at 4°C, remove supernatant
3. Resuspend the two pellets with 10mL Resuspension Buffer (R3) in one 250mL centrifuge bottle
4. Add 10mL Lysis Buffer (L7), mix gently by inverting the bottle, incubate at room temperature for 5mins
5. Add 10mL Precipitation Buffer (N3), mix immediately but gently by inverting the bottle
6. Centrifuge at ≥20,000 x g for 5 min at 4°C
7. Place the MIDI column (with filtration cartridge) on the column holder with a tray to collect the flow through waste
8. Add 15mL Equilibration buffer EQ1 to the column (10mL first, wait for flow through, then add another 5mL)
9. Pull all the supernatant after **step 6** to the MIDI column (with filtration cartridge) and wait for complete flow through
10. Remove the filtration cartridge from the MIDI column, add 10mL Wash buffer (W8) to the column and wait for complete flow through
11. Again, add 20mL **Wash buffer (W8)** to the column and wait for complete flow through
12. Add 3.5mL **99% Isopropanol** to the falcon

13. Place the column to the falcon, add 5mL **Elution buffer (E4)** to the column, wait for complete elute to the falcon
14. **Mix** the eluent with 99% Isopropanol well
15. Centrifuge at 6000x g for 1 hour at 4°C
16. Remove all supernatant, add 3mL **70% Ethanol** to resuspend the DNA pellet
17. Transfer the DNA pellet together with 70% Ethanol to a new 1.5mL eppendorf
18. Centrifuge at 16,000 g for 1min at room temperature
19. Remove all 70% Ethanol from the 1.5mL eppendorf
20. Repeat step 13-15 until all the DNA with 70% Ethanol is transferred from Falcon to the 1.5mL eppendorf
21. Dried the DNA pellet on 50°C heat block (around several seconds to 1min), be careful **NOT** completely dried the DNA pellet (completely transparent)
22. Add ~200µL **pre-warmed 50°C ddH₂O** to dissolve the DNA pellet (warm on 55°C heat block with occasionally vortex)
23. Proceed to Nano Drop to measure the DNA concentration and purity, store this plasmid DNA at -20°C

MV-V	2.97ug/uL
Orf6 WT	3.395ug/uL
Orf6 Omi	1.83ug/uL
Orf9b WT	3.395ug/uL
Orf9b Delta	1.943ug/uL
Orf9b Omi	1.83ug/uL

Send sequencing

Send 250ng plasmid to Sanger Sequencing

Transform ISRE-Luc plasmid & pPL-TK plasmid into DH5α

10/6/2022

1. Thaw the competent cell (DH5α *E.coli*) on ice and also place the ligation product on ice for at least 1min
2. Add 50µL competent cell to the 1uL plasmid , flap the tubes to brief mixing (Do not vortex!!), and place back on ice for 20mins
3. Heat shock the competent cell – ligation product mix on 42°C water bath or heat block for 1 min
4. Place back on ice immediately for 5mins
5. Add 500µL plain LB broth to the tubes and incubate at 37°C incubator with shaking for 1hr (Amp resistant)
6. Centrifuge the bacteria at max speed for 1min

7. Remove the supernatant to ~50µL LB broth left in the tubes
8. Re-suspend the bacteria with this ~50µL LB and spread the bacteria to LB (Amp) plate
9. Incubate at 37°C incubator for 12-16 hours overnight

Pick colonies

11/6/2022

1. Aliquot 500µL LB(Amp) to a 1.5mL eppendorf
2. Pick 1 colony of each reaction to the corresponding 500µL growth medium
3. Incubate at 37°C incubator for ~6 hours with shaking

Subculture for Midi-prep

13/6/2022

- ISRE-Luc colony 1
 - pPL-TK colony 1
1. Add 50uL bacteria to 50mL LB(Amp)
 2. Incubate at 37°C, 250rpm for 12-16 hours

Midi-prep

14/6/2022

1. Pour 50mL growth overnight culture in a 50mL centrifuge tube
2. Centrifuge at 6000x g for 15mins at 4°C, remove supernatant
3. Resuspend the two pellets with 10mL Resuspension Buffer (R3) in one 250mL centrifuge bottle
4. Add 10mL Lysis Buffer (L7), mix gently by inverting the bottle, incubate at room temperature for 5mins
5. Add 10mL Precipitation Buffer (N3), mix immediately but gently by inverting the bottle
6. Centrifuge at ≥20,000 x g for 5 min at 4°C
7. Place the MIDI column (with filtration cartridge) on the column holder with a tray to collect the flow through waste
8. Add 15mL Equilibration buffer EQ1 to the column (10mL first, wait for flow through, then add another 5mL)
9. Pull all the supernatant after **step 6** to the MIDI column (with filtration cartridge) and wait for complete flow through

10. Remove the filtration cartridge from the MIDI column, add 10mL **Wash buffer (W8)** to the column and wait for complete flow through
11. Again, add 20mL **Wash buffer (W8)** to the column and wait for complete flow through
12. Add 3.5mL **99% Isopropanol** to the falcon
13. Place the column to the falcon, add 5mL **Elution buffer (E4)** to the column, wait for complete elute to the falcon
14. **Mix** the eluent with 99% Isopropanol well
15. Centrifuge at 6000x g for 1 hour at 4°C
16. Remove all supernatant, add 3mL **70% Ethanol** to resuspend the DNA pellet
17. Transfer the DNA pellet together with 70% Ethanol to a new 1.5mL eppendorf
18. Centrifuge at 16,000 g for 1min at room temperature
19. Remove all 70% Ethanol from the 1.5mL eppendorf
20. Repeat step 13-15 until all the DNA with 70% Ethanol is transferred from Falcon to the 1.5mL eppendorf
21. Dried the DNA pellet on 50°C heat block (around several seconds to 1min), be careful **NOT** completely dried the DNA pellet (completely transparent)
22. Add **~200µL pre-warmed 50°C ddH₂O** to dissolve the DNA pellet (warm on 55°C heat block with occasionally vortex)
23. Proceed to Nano Drop to measure the DNA concentration and purity, store this plasmid DNA at -20°C

ISRE-Luc	1.332 ug/uL
PL-TK	777.5 ng/uL

Luciferase Assay

(1) Pass Cell (293-FT)

11/6/2022

1. Use suction & glass pipette to remove all the medium in the plate
2. Add **1mL Trypsin** and incubate for **3 mins**
3. Add **6mL medium (with FBS)** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
4. Collect to a 14mL centrifuge tube → pipette up & down
5. Centrifuge for **5mins**, room temp, 1000 rpm
6. At the same time, take a new plate, add **9mL medium** (with FBS) to it X2
7. After centrifugation, use suction and glass pipette to remove all the supernatant in the centrifuge tube
8. Add **5mL (1:5)** medium to resuspend the pellet of VeroE6 TMPRSS2 and BHK21 respectively
9. Add **1mL** cell culture to the new plate X2 --> 10cm dish X2

(2) Seed cell (293FT) in 24 well plate X 2

15/6/2022

1. Remove supernatant
2. Add 1mL Trypsin
3. Incubate 3 min
4. Add 6mL DMEM medium to neutralize resuspend Transfer to 15/50mL falcon
5. Centrifuge at 1000rpm for 5 min at r.t
6. Remove supernatant
7. Resuspend the cell pellet with 10mL DMEM medium

8. Count Cell

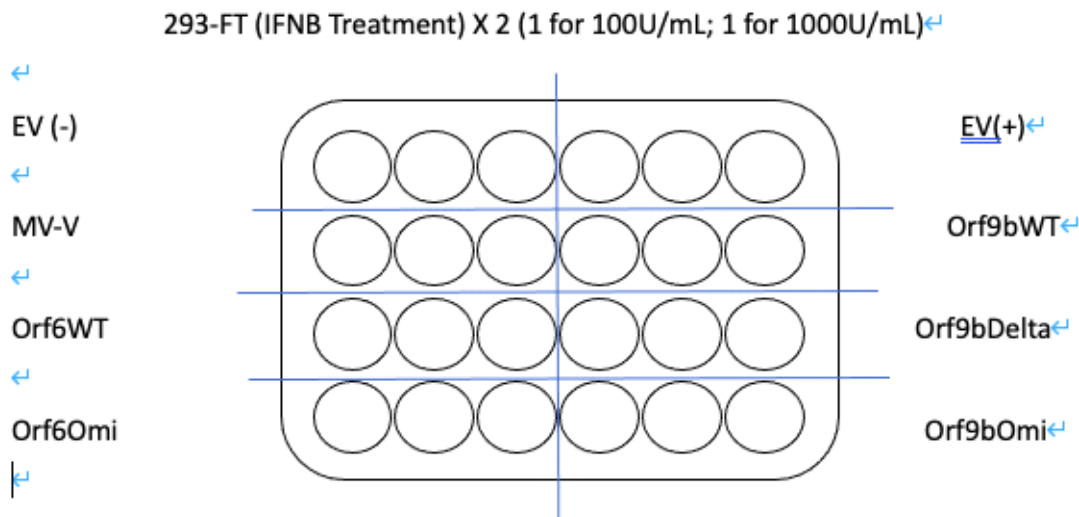
- Target: **1.2×10^5 cells/well** (500uL medium per well)
 0.12×10^6 per well
 0.24×10^6 per mL
Total: 72 well ~ 75well
 $0.5\text{mL} \times 75 \text{ well} = \mathbf{37.5 \text{ mL}}$
Need: 0.24×10^6 cells per mL $\times 37.5 \text{ mL} = \mathbf{9 \times 10^6}$ cells

- Count cell: **152**
 $152 / 4 \times 4 (\text{dilution factor}) = 152 \times 10^4 = \mathbf{1.52 \times 10^6}$

$$\frac{9 \times 10^6 \text{ cells}}{1.52 \times 10^6} \\ = \mathbf{5.92 \text{ mL}}$$

- Mastermix: **5.92 mL** cell + 31.58mL DMEM

9. Add **500uL** to each well



(3) Transfection

16/6/2022

- Transfection Mastermix (3.5X):
Dilute plasmids into working concentration:
orfs & ISRE-Luc 100ng/uL; pRL-TK: 10ng
500ng plasmid per well:
ISRE-Luc: 100ng; pRL-TK: 10ng ; orf: 385ng
Mastermix (ISRE-Luc + pRL-TK) = $1\mu\text{L} \times 3.5 \times 20 + 1\mu\text{L} \times 3.5 \times 20 = 140\mu\text{L}$

IFNB activation:

IFNB activation								
	EV (-ve)	EV (+ve)	MV-V	Orf6 WT	Orf6 Omik	Orf9b WT	Orf9b Delta	Orf9b Omik
Opti-MEM	66.85uL							
Plasmid	13.5uL							
ISRE-Luc + pRL-TK	7uL							
Total	87.5uL							

→ Mix well in 1mL Eppendorf (Opti-MEM-DNA + Opti-MEM-GeneJuice)
[DO NOT VORTEX!!!] **Total: 50uL per well**

- Incubate at r.t. for **20-25min**
- Remove 50uL DMEM medium from each well
- Add the **50uL Transfection Mastermix** into each well dropwise

(4) Treat IFNB (24hrs post-transfection)

17/6/2022

- Mastermix: 1uL IFNB + 10mL DMEM medium (1:10000)

Serial dilution: **2uL IFNB** in **1998uL DMEM** (1:1000)

→ **1.7mL** in **15.3mL DMEM** (1:10000) = 1000U/mL

→ **1.5mL** in **13.5mL DMEM** = 100U/mL

- Remove supernatant from each well
- Add 500uL into each well

(5) Measure Luciferase Activity (12 hours post-treatment)

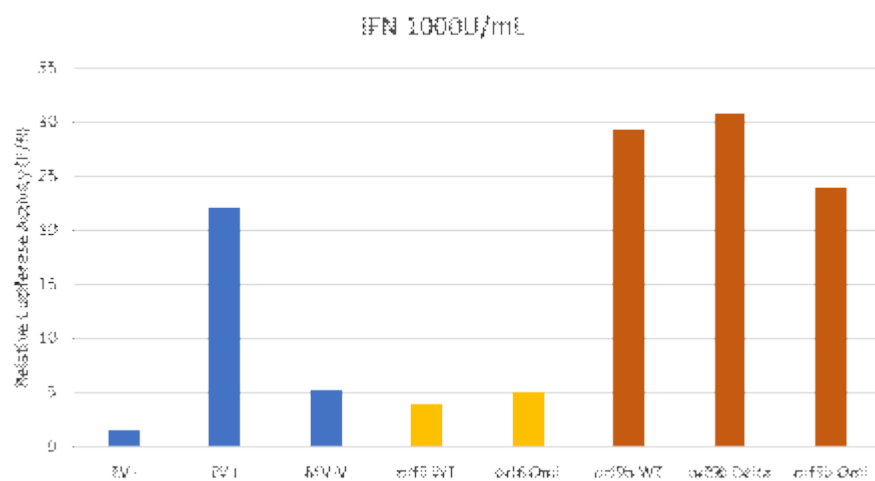
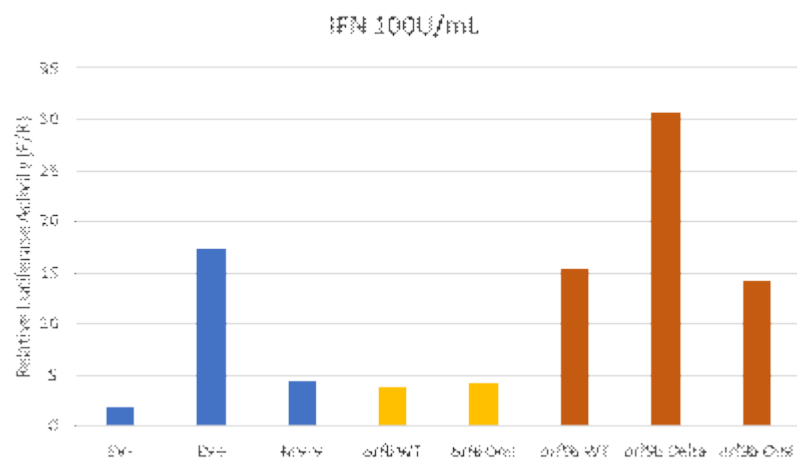
17/6/2022

- Dilute 5X lysis buffer into 1X
- 1mL lysis buffer + 4mL ddH₂O

3. Remove supernatant
4. Lyse cell by adding **100uL 1X lysis buffer** per well
5. Shake for 30min at r.t
6. Transfer **10uL lysate** to white luciferase plate
7. Add **50uL 1:1 diluted LARII** per well
8. Read the result on plate reader (**firefly**) --> excel
9. Add **50uL 1:1 diluted Stop & Glow with substrate (1:100)** per well
10. Read the result on plate reader (**renilla**) --> excel
11. **Calculation: firefly / renilla**

	IFN (100U/mL)
EV-	1.892
EV+	17.432
MV-V	4.472
orf6 WT	3.792
orf6 Omi	4.231
orf9b WT	15.479
orf9b Delta	30.628
orf9b Omi	14.313

	IFN (1000U/mL)
EV-	1.571
EV+	22.038
MV-V	5.357
orf6 WT	3.983
orf6 Omi	5.077
orf9b WT	29.241
orf9b Delta	30.737
orf9b Omi	23.976



12. Plot graph by Prism (Calculate fold change)

Viral growth kinetics

2023年6月27日星期二 下午6:23

Seed cell (VeroE6 & A549-ACE2-TMPRSS2) in P2 lab

15/4/2022

1. Use suction & glass pipette to remove all the medium in the plate (6X10cm dish)
2. Add **1.5mL PBS** to wash the cell for twice
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins**
5. Add **6mL DMEM +10%FBS** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect into 2X 50mL centrifuge tubes → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 10mL DMEM+10% X2

Count cell:

VeroE6 Target: 1.5×10^5 /well

A549-ACE2-TMPRSS2: 2×10^5 /well

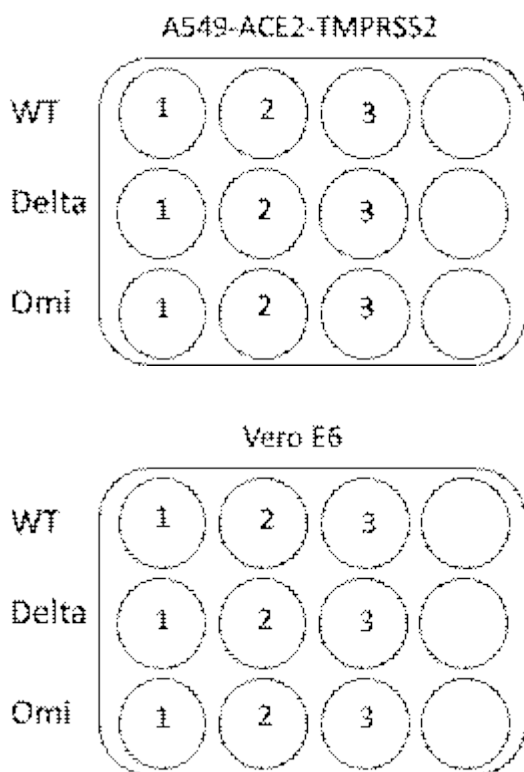
10. Add 1mL to each well of 12-well plate (Total: 6 plate)

Infection in P3 lab

16/4/2022

1. Remove all medium of fresh cell

2. Transfer 1mL DMEM to fresh cell
3. Inoculate virus (MOI = 0.01) and incubate for 1 hr
4. Remove all medium
5. Wash with 0.5mL PBS of each well twice
6. Replace 1mL DMEM+1% FBS



Harvest supernatant (12hrs, 24hrs, 48hrs post-infection) in P3 lab

17/4/2022; 18/4/2022

1. At the corresponding timepoint, transfer viral supernatant of each well to a 14mL falcon
2. Centrifuge at 14mL falcon for 5 min at MAX speed
3. Transfer supernatant to 1.5mL screw cap.
4. Transfer 140uL viral supernatant of each sample to a 1.5mL screw cap containing 560uL AVL buffer respectively.

AVL to RNA

19/4/2022

1. 140uL viral supernatant and 560uL AVL Buffer and mixed by vortex.
2. The viral particles were lysed after 10min incubation at room temperature.
3. 560uL absolute ethanol was added and mixed by pulse-vortexing for 15 minutes.
4. 630uL of each sample were transferred QIAamp Mini column and centrifuged at 6000xg for 1 minute at room temperature.
5. Place the QIAamp Mini column into a clean collection tube.
6. Repeat once to let all sample pass through the filter.
7. Add 500uL Buffer AW1 to each column and centrifuged at 6000xg for 1 minute at room temperature.
8. Place the QIAamp Mini column into a clean collection tube.
9. Add 500uL Buffer AW2 and centrifuged at 20000xg for 3 minutes at room temperature.
10. Place the QIAamp Mini column into a clean 1.5mL Eppendorf
11. Add 30uL Buffer AVE to the column and incubate at room temperature for 1 minute
12. Centrifuge at 6000xg at room temperature

Reverse Transcription (RNA to cDNA)

1. gDNA erase:

Reagent	Amount <Per reaction>
5X gDNA Eraser Buffer	2.0 µL
gDNA Eraser	1.0 µL
RNA	<u>7 µL</u> RNA from AVL viral supernatant RNA extraction
Total	10.0 µL

Incubate at Thermal cycler

Temperature	Duration

42°C	2mins
4°C	Forever

2. RT:

Reagent	Viral supernatant RNA
	Amount (Gene specific primer) [per reaction]
5X PrimeScript Buffer 2 (for Real Time)	4.0 µL
PrimeScript RT Enzyme Mix I	1.0 µL
Primer	0.5 µL RT-2019-nCoV_3UTR
RNase Free dH ₂ O	4.5 µL
Total	10.0 µL

Incubate at Thermal cycler

Temperature	Duration
42°C for Gene specific primer	60 min.
85°C	5 sec.
4°C	Forever

3. cDNA product can be stored at -20°C for long term storage
4. Aliquot some cDNA and make 2x dilution for qPCR
5. Use 2 µL for each qPCR reaction

qPCR

20/4/2022

1. For each SYBR® Green reaction:

	Amount
SYBR Premix Ex Taq (Tli RNaseH Plus) (2X)	5 µL
Primer set	0.5 µL Forward primer (2.5 µM)
	0.5 µL Reverse primer (2.5 µM)
ROX Reference Dye (50X) or Dye II (50X)	0.2 µL
Template	2µL dilute cDNA
DDH₂O	1.8 µL

2. Add 2uL diluted cDNA and 8uL mastermix solution to 385-well plate
3. Real time cycler setting (ViiA7):

OpenStudy™ Path-Flow Software v.2
 File Edit View Help
 Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent
 Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent Open Recent

Experiment: 2022-01-17 14:58:11_P02_C0A2_1002A_00 Type: Comparative C [MAN] Reagents: SYBR® Green Reagents

How do you want to identify this experiment?

Experiment name: 2022-01-17 14:58:11_P02_C0A2_1002A_00 Comments:

Barcode: Date taken:

Which instruments type are you using to run this experiment?

Quantification: qPCR System Quantification: qPCR System ☒ **qPCR / System**

Which blocks are you using to run this experiment?

☒ **SYBR** ☐ **Real-time** ☐ **Real-time** ☐ **Real-time** ☐ **Real-time**

What type of experiment do you want to set up?

☐ Standard Curve ☐ Relative Quantification ☒ **Comparative C [MAN]** ☐ Real-time

Which reagents do you want to use to detect the target sequence?

☒ **SYBR® Green Reagents** ☐ **Real-time**

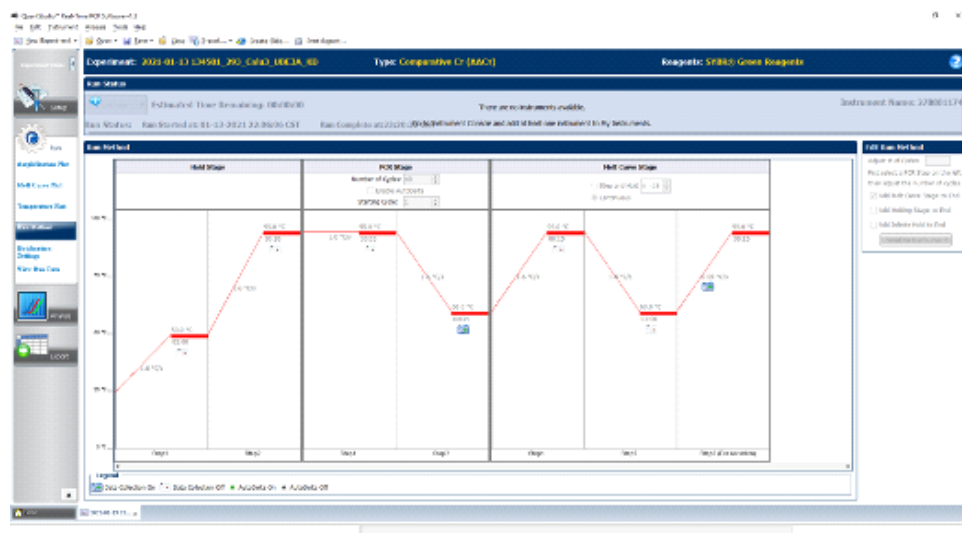
What properties do you want for the instrument run?

☒ **Standard** ☐ **Real-time**

What is the reagent information?

Run: Date:

Type: Name: Path: Path: Path: Path:



Seed cell (VeroE6 & A549-ACE2-TMPRSS2) in P2 lab

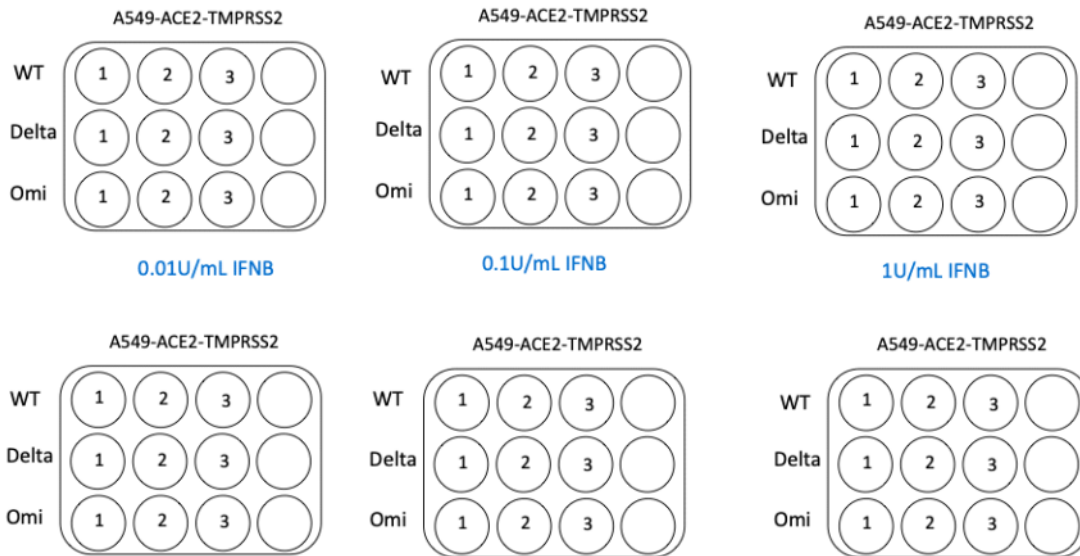
28/4/2022

1. Use suction & glass pipette to remove all the medium in the plate (6X15cm dish)
2. Add **1.5mL PBS** to wash the cell for twice
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins**
5. Add **6mL DMEM +10%FBS** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect into 2X 50mL centrifuge tubes → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 10mL DMEM+10% X2

Count cell:

A549-ACE2-TMPRSS2: 2×10^5 /well

10. Add 1mL to each well of 12-well plate (Total: 12 plate)



Treat IFNB (24hrs post-seed cell) in P2 lab

29/4/2022

1. Mastermix: 1uL IFNB + 10mL DMEM medium (1:10000)

Serial dilution: 2uL IFNB in 1998uL DMEM (1:1000)

- 2 mL in 18 mL DMEM (1:10000) = 1000U/mL
- 2 mL in 18 mL DMEM = 100U/mL
- 2 mL in 18 mL DMEM = 10U/mL
- 2 mL in 18 mL DMEM = 1U/mL
- 2 mL in 18 mL DMEM = 0.1U/mL
- 2 mL in 18 mL DMEM = 0.01U/mL

2. Remove supernatant from each well
3. Add 1mL into each well

Infection (12hrs post-treatment) in P3 lab

30/4/2022

1. Remove all medium of fresh cell
2. Transfer 1mL DMEM to fresh cell
3. Inoculate virus (1000 PFU WT/Delta/Omi) and incubate for 1 hr

4. Remove all medium
5. Wash with 0.5mL PBS of each well twice
6. Replace 1mL DMEM+1% FBS

Harvest Supernatant (12 hours and 48 hours post-infection)

30/4/2022; 2/5/2022

1. At the corresponding timepoint, transfer viral supernatant of each well to a 14mL falcon
2. Centrifuge at 14mL falcon for 5 min at MAX speed
3. Transfer supernatant to 1.5mL screw cap.

Seed cell (VeroE6) in P2 lab

2/5/2022

1. Use suction & glass pipette to remove all the medium in the plate (6X15cm dish)
2. Add **1.5mL PBS** to wash the cell for twice
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins**
5. Add **6mL DMEM +10%FBS** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect into 2X 50mL centrifuge tubes → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 10mL DMEM+10% X2

Count cell:

VeroE6: 1.5×10^5 / well

10. Add _____ to each well of 96-well black plate

FFU in P3 lab

3/5/2022

1. Inoculate 50uL viral supernatant to each well of VeroE6 cells for 1 hour at 37°C
2. Discard medium and replaced by DMEM+1%FBS

4/5/2022

3. After 12 hours incubation, add 4% PFA to fix the cell overnight

5/5/2022

4. Permeabilized with 0.1% NP-40
5. Block with 2% BSA
6. Immunostained for NP-positive cells using rabbit in-house antibodies
7. Nucleocapsid protein positive cells were counted under fluorescent microscope and using Cytation7
8. Plot IC-50 curves by Prism

IFN profiling

2023年6月28日星期三 下午6:35

Seed cell (A549-ACE2-TMPRSS2)

9/5/2022

1. Use suction & glass pipette to remove all the medium in the plate (10cm dish)
2. Add **1.5mL PBS** to wash the cell for twice
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins**
5. Add **6mL DMEM +10%FBS** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect into 2X 50mL centrifuge tubes → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 10mL DMEM+10%
10. Count cell:
Target: 2×10^5 /well
11. Add 1mL to each well of 12-well plate

Treat IFNB (12hrs post-seed cell)

10/5/2022

1. Mastermix: 1uL IFNB + 10mL DMEM medium (1:10000)

Serial dilution: **2uL** IFNB in **1998uL** DMEM (1:1000)

→ **1.7mL** in **15.3mL** DMEM (1:10000) = 1000U/mL

2. Remove supernatant from each well
3. Add 1mL into each well

Harvest cell & Extract RNA (Trizol)

11/5/2022

1. Discard cell supernatant by suction
2. Add 1mL Trizol reagent to the wells and Incubate at room temp. for 5mins
3. Transfer to eppendorf
4. Add 0.2mL Chloroform (per 1mL Trizol)
5. Vortex for 1min
6. Spin down at 12,000g for 15mins at 4°C
7. Prepare a new eppendrof containing 500µL 100% Isopropanol (per 1mL Trizol)
8. Transfer the aqueous phase (upper) of the sample after spin down and dropwise addition tom the 500µL 100% Isopropanol
9. Hand mixing to homogeneous
10. Incubate at room temp. for 10mins
11. Centrifuge at 12,000g for 10mins at 4°C
12. Remove all supernatant, add 1mL 75% EtOH (per 1mL Trizol)
13. Briefly vortex, then centrifuge at 7,500g for 5mins at 4°C
14. Remove all supernatant, allow air dry the pellet for 5-10mins (**not fully dry)
15. Re-suspend the pellet with 10-20µL ddH₂O.

Reverse Transcription (RNA to cDNA)

16. gDNA erase:

Reagent	Amount <Per reaction>
5X gDNA Eraser Buffer	2.0 µL

gDNA Eraser	1.0 μ L
RNA	1ug RNA
Total	10.0 μL

Incubate at Thermal cycler

Temperature	Duration
42°C	2mins
4°C	Forever

17. RT:

Reagent	Infected cell RNA
	Amount (RT primer mix) [per reaction]
5X PrimeScript Buffer 2 (for Real Time)	4.0 μ L
PrimeScript RT Enzyme Mix I	1.0 μ L
Primer	1.0 μ L (RT primer mix)
RNase Free dH ₂ O	4.0 μ L
Total	10.0 μL

Incubate at Thermal cycler

Temperature	Duration
37°C for RT primer mix	15 min.
85°C	5 sec.
4°C	Forever

18. cDNA product can be stored at -20°C for long term storage

19. Aliquot some cDNA and make 2x dilution for qPCR

20. Use 2 μ L for each qPCR reaction

qPCR

12/5/2022

1. For each SYBR® Green reaction:

	Amount

SYBR Premix Ex Taq (Tli RNaseH Plus) (2X)	5 µL
Primer set	0.5 µL Forward primer (2.5 µM)
	0.5 µL Reverse primer (2.5 µM)
ROX Reference Dye (50X) or Dye II (50X)	0.2 µL
Template	2µL dilute cDNA
DDH₂O	1.8 µL
Total	20 µL

Primer:

hIFT1	hIFT1-F
	hIFT1-R
hMx1	hMx1-F
	hMx1-R
hOAS1	hOAS1-F
	hOAS1-R
hOAS2	hOAS2-F
	hOAS2-R
ISG15	ISG15-F
	ISG15-R
IFI6	IFI6-F
	IFI6-R
IFITm1	IFITm1-F
	IFITm1-R
IFITm3	IFITm3-F
	IFITm3-R
RIG-I	RIG-I-F
	RIG-I-R
MDA5	MDA5-F
	MDA5-R
IP-10	IP-10-F
	IP-10-R
RANTES	RANTES-F
	RANTES-R

2. Add 2uL diluted cDNA and 8uL mastermix solution to 385-well plate
3. Real time cycler setting (ViiA7):

PCR Amplification of Kan-I-SceI

Dilute the primers for PCR Amplification (BAC Recombination)

26/10/2021

- Add **40uL autoclaved ddH₂O** (4nmol of primer)
- Vortex & Spin down twice
- 50°C Heat block for around 1 min
- Vortex & Spin down
- Diluted 1:10 (10uL Primer + 90uL autoclaved ddH₂O)

PCR Reaction:

1/11/2021

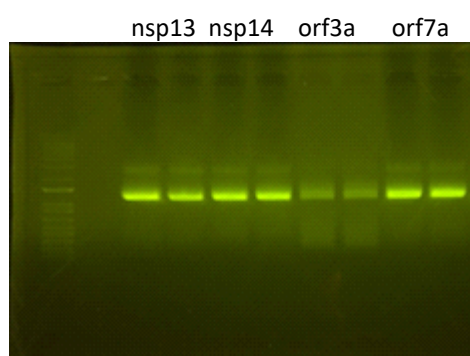
	SARS2-nsp13-P77L (2X)
5X KAPA HiFi Buffer (Fidelity)	10 µL
Primer set	1.5 µL SARS2-nsp13-P77L-recom-F (10µM)
	1.5 µL SARS2-nsp13-P77L-recom-R (10µM)
dNTP	1.5 µL
Template	2.0 µL (10ng/µL) pEPkan-S
KAPA HiFi	1.0 µL
DDH₂O	32.5 µL
Total	50 µL

	SARS2-orf3a-S26L (2X)
5X KAPA HiFi Buffer (Fidelity)	10 µL
Primer set	1.5 µL SARS2-orf3a-S26L-recom-F (10µM)
	1.5 µL SARS2-orf3a-S26L-recom-R (10µM)
dNTP	1.5 µL
Template	2.0 µL (10ng/µL) pEPkan-S
KAPA HiFi	1.0 µL
DDH₂O	32.5 µL
Total	50 µL

	SARS2-orf7a-T120I (2X)
5X KAPA HiFi Buffer (Fidelity)	10 µL
Primer set	1.5 µL SARS2-orf7a-T120I-recom-F (10µM)
	1.5 µL SARS2-orf7a-T120I-recom-R (10µM)
dNTP	1.5 µL
Template	2.0 µL (10ng/µL) pEPkan-S
KAPA HiFi	1.0 µL
DDH₂O	32.5 µL
Total	50 µL

PCR setting:

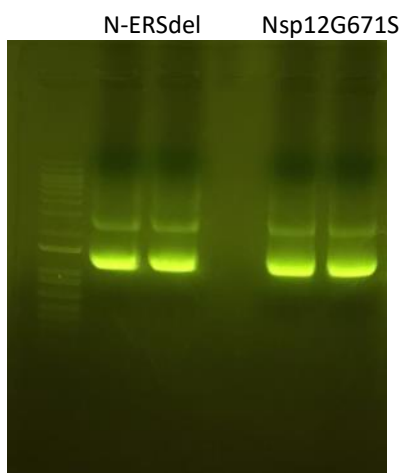
		Temperature	Time
Initial denature		95 °C	3mins
Cycling (30 cycles)	Denature	98 °C	20s
	Annealing	56 °C	15s
	Extension	72 °C	1 min
Final Extension		72 °C	5mins
Store		4 °C	∞



	SARS2 NSP12 G671S
5X KAPA HiFi Buffer (Fidelity)	10 µL
Primer set	1.5 µL SARS2-nsp12-G671S-recom-F (10µM)
	1.5 µL SARS2-nsp12-G671S-recom-R (10µM)
dNTP	1.5 µL
Template	2 µL pEPkan-S (100ng/uL)

KAPA HiFi	1 µL
DDH ₂ O	32.5 µL
Total	50 µL

	SARS2-N-ERS del
5X KAPA HiFi Buffer (Fidelity)	10 µL
Primer set	1.5 µL SARS2-N-ENAdel-recom-F (10µM)
	1.5 µL SARS2-N-ENAdel-recom-R (10µM)
dNTP	1.5 µL
Template	2 µL pEPkan-S (100ng/uL)
KAPA HiFi	1 µL
DDH ₂ O	32.5 µL
Total	50 µL



PCR setting:

		Temperature	Time
Initial denature		95 °C	3mins
Cycling (30 cycles)	Denature	98 °C	20s
	Annealing	56 °C	15s
	Extension	72 °C	1 min
Final Extension		72 °C	5mins
Store		4 °C	∞

Agarose Gel electrophoresis :

1. Prepare 25mL 1% agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
2. Cool the gel solution a little bit, then add 1.25 μ L (1:20000) SmartGlow to the gel solution and mix well
3. Pour the gel to the gel rack and wait for solidification.
4. Transfer the gel to the gel tank soaked with 1X TAE buffer
5. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
6. Run the gel at 120V (constant voltage) for 24mins.
7. Visualize the gel under Blue box and cut the DNA band to a 1.5mL eppendrof (better to keep gel slice below 0.2 g)

Gel Purification (GeneJET Gel Extraction and DNA Cleanup Micro Kit)

1. Add **200 μ L** of **Extraction Buffer** to the eppendrof containing the
2. Heat at 50°C on the heat block for 10 minutes or until the gel slice is completely dissolved
3. Add **200 μ L** of **absolute ethanol (96-100%)** and mix by pipetting
4. Transfer the mixture to the DNA Purification Micro Column (white) preassembled with a collection tube.
5. Centrifuge the column at 20,817 \times g (max speed) for 1min
6. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
7. Add **200 μ L** of **Prewash Buffer** (supplemented with ethanol) to the column
8. Centrifuge the column at 20,817 \times g (max speed) for 1min
9. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
10. Add **700 μ L** of **Wash Buffer** (supplemented with ethanol) to the column
11. Centrifuge the column at 20,817 \times g (max speed) for 1min
12. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.

13. Add **700 µL of Wash Buffer** (supplemented with ethanol) to the column
14. Centrifuge the column at $20,817 \times g$ (max speed) for 1min
15. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
16. Centrifuge the column at $20,817 \times g$ (max speed) for addition 1min to completely remove residual Wash Buffer
17. Transfer the DNA Purification Micro Column into a clean 1.5 mL eppendrof
18. Add **20 µL of warmed ddH₂O** to the DNA Purification Micro Column.
19. Centrifuge at $20,817 \times g$ (max speed) for 1min to elute DNA

Nano Drop measurement

1. Open the software and click “Nucleic acid”
2. Pipette 10µL ddH₂O and add ~1µL to each detector head (total 8 heads)
3. Click “OK” to initiate the Nano Drop equipment motor
4. Select “DNA-50”, then select all detector heads to “Blank”
5. Remove the solvent from all detector heads
6. Add 1µL DNA sample to the detector head, checked the corresponding detector head for measurement and click “Measure”
7. Record the DNA concentration in ng/µL and the purity

Nsp13 P77L	127 ng/uL
Nsp14 A394V	161.8 ng/uL
Orf3a S26L	33.17 ng/uL --> Re-amplification: 75.55 ng/uL
Orf7a T120I	165.8 ng/uL
Nsp12 G671S	182.6 ng/uL
N ERS del	184.5 ng/uL

1st round recombination

(1) Transform SARS2 WT Bac Plasmid to GS1783

1. Thaw the SW105 bacteria on ice
2. Add **1uL** of **pBAC-SARS-CoV-2-WT** to the GS1783 cell, flick the tubes to brief mixing (Do not vortex!!), transfer the mixture to the electroporation cuvette (0.2cm gap) and place back on ice for **10mins**
3. Initiate an electrical pulse of **2.5kV**, 200 Ω , 25 μ F at a time constant of >4.1ms (ideally is 5.0ms)
4. Immediately add 1mL plain LB broth to the electroporation cuvette (don't flush too hard), then transfer to a 1.5mL eppendorf and incubate at **32°C, 220rpm for 1hr**
5. Spin down the bacteria, remove the supernatant until 100uL left
6. Spread **all the bacteria** to the **LB(Cm)** plate
7. Incubate at 32°C incubator for 16-18hrs

(2) Pick clone and inoculation of recombinant GS1783

1. Aliquot **1mL LB(Cm)** falcon each
2. Pick one isolated colony to the corresponding 1mL growth medium
3. Incubate at **32°C**, 220rpm (6-8 hours)
4. Add 1uL culture grown for ~6hrs to 1mL LB (Cm) medium (1:1000 inoculum)
5. Incubate at **32°C**, 220rpm overnight (16-18 hours)

(3) Prepare competent cell (GS1783 SARS2 CoV-2 WT)

1. Inoculate 150uL overnight grown culture into 7.5mL LB(Cm) (1:50) for each reaction

2. Shake at 32°C until OD600 reaches 0.5-0.7 (~4 hrs)
3. Transfer bacteria to 50mL falcon and shake in 42°C water bath for 15 min
4. Chill bacteria for 20 min in ice bath
5. Spin bacteria for 5min at 1°C, 4500g
6. Remove supernatant and resuspend pellet in 1mL 10% ice-cold glycerol and transfer to 2mL eppendorf
7. Spin bacteria for 1min at 1°C, 12000g
8. Discard supernatant and repeat the washing step
9. Resuspend bacteria with 50uL 10% ice-cold glycerol

(3) 1st Round Recombination

1. Add 100ng PCR product to 50uL **GS1783 SARS2 CoV-2 WT** competent cell
2. Transfer the culture to chilled 1mm electroporation cuvette
3. Electroporate at 1.5kV
4. Add 1mL plain LB and transfer to eppendorf
5. Shake the bacteria culture for 1.5hr
6. Spread LB(Kan, Cm) plate and incubate at 32°C for 24hrs

(4) Pick colonies and subculture for PCR Screening

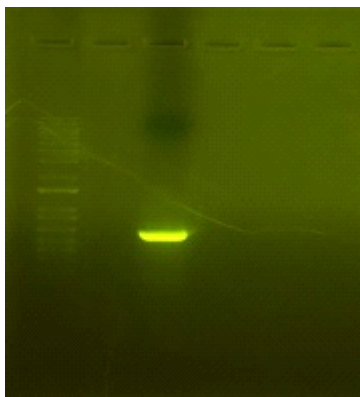
No colonies for NSP14 A394V (FAILED)!

1. Aliquot 1mL LB(Cm,Kan) a 14mL falcons
2. Pick isolated colony to the corresponding 1mL growth medium
3. Incubate at 32°C, 220rpm for 16-18hrs overnight

PCR Screening after 1st round recombination

	SARS2-nsp13-P77L
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL nCoV-NSP12-F2741
	0.8 µL pEPkan-S-kan_R256
dNTP	0.4 µL
Template	1uL overnight grown culture
Dream Taq	0.1 µL
DDH ₂ O	14.9 µL
Total	20 µL

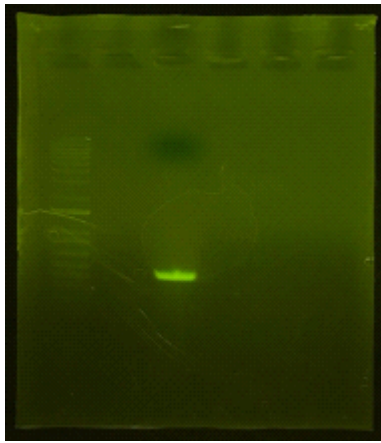
Target size: 574bp



correct

	SARS2-Orf3a-S26L
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL nCoV-Spike-F3755
	0.8 µL pEPkan-S-kan_R256
dNTP	0.4 µL
Template (overnight grew culture)	1 µL
Dream Taq	0.1 µL
DDH ₂ O	14.9 µL
Total	20 µL

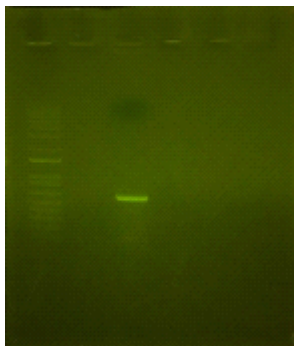
Target size: 403 bp



correct

	SARS2-ORF7a-T120I
Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L nCoV-ORF7a-F21
	0.8 μ L pEPkan-S-kan_R256
dNTP	0.4 μ L
Template	colony
Dream Taq	0.1 μ L
DDH ₂ O	15.9 μ L
Total	20 μ L

Target size: 602bp



correct

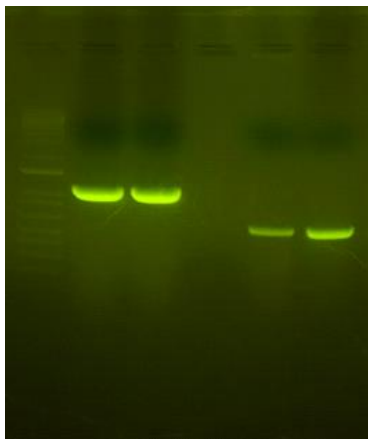
	NSP12 G671S
Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L pEPkan-S-kan_F840
	0.8 μ L nCoV-NSP12-F1235
dNTP	0.4 μ L
Template	1uL overnight grown culture

Dream Taq	0.1 µL
DDH₂O	14.9 µL
Total	20 µL

Target size: 1063bp

	N ENA del
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL pEPkan-S-kan_F840
	0.8 µL nCoV-Nuc-R435
dNTP	0.4 µL
Template	1uL overnight grown culture
Dream Taq	0.1 µL
DDH₂O	14.9 µL
Total	20 µL

Target size: 569bp



All correct

PCR setting

		Temperature	Time
Initial denature		95 °C	3mins
Cycling (30 cycles)	Denature	95 °C	30s
	Annealing	55 °C	30s
	Extensiton	72 °C	1min (2kb/1min, then 1kb/min)
Final Extension		72 °C	5mins
Store		4 °C	∞

Agarose Gel electrophoresis :

1. Prepare 25mL 1% agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
2. Cool the gel solution a little bit, then add 1.25 μ L (1:20000) SmartGlow to the gel solution and mix well
3. Pour the gel to the gel rack and wait for solidification.
4. Transfer the gel to the gel tank soaked with 1X TAE buffer
5. Add 10 μ L 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
6. Run the gel at 120V (constant voltage) for 24mins.
7. Visualize the gel under Blue box

2nd round recombination

(1) 2nd Round Recombination

1. Add 20uL overnight grown culture to 1mL LB(Cm) and shake at 32°C for 2 hrs
2. 2% arabinose= 0.1g + 5mL LB(Cm)
3. Add 1mL LB(Cm, 2% ara) and shake for 1 hr
4. Shake at 42°C water bath for 30min
5. Shake at 32°C for 2 hrs
6. Dilute **1:10000** and spread 100uL bacteria to LB(Cm, 1% ara) plate

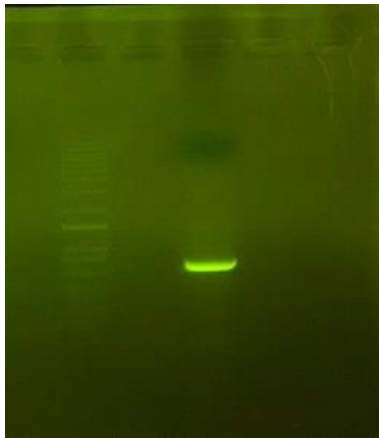
(2) Pick colonies and subculture for PCR Screening

1. Aliquot 1mL LB(Cm) a 14mL falcons
2. Pick isolated colony to the corresponding 1mL growth medium
3. Incubate at 32°C, 220rpm for 6-8hrs

PCR Screening after 2nd round recombination

	SARS2-nsp13-P77L
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL nCoV-NSP12-F2741
	0.8 µL nCoV-NSP13-R604
dNTP	0.4 µL
Template (overnight grew culture)	1 µL
Dream Taq	0.1 µL
DDH₂O	14.9 µL
Total	20 µL

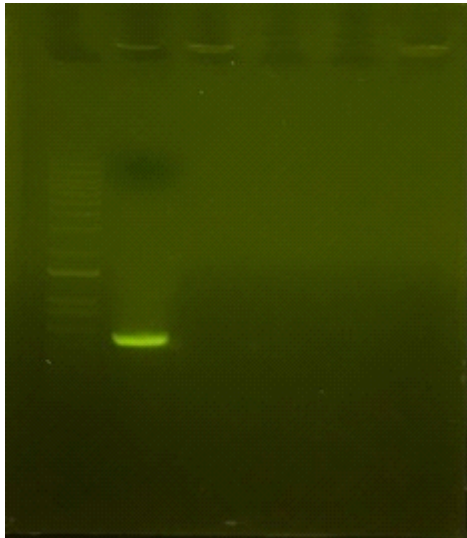
Target size: 703bp



Correct

	SARS2-Orf3a-S26L
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL nCoV-Spike-F3755
	0.8 µL nCoV-ORF3a-R425
dNTP	0.4 µL
Template (overnight grew culture)	1 µL
Dream Taq	0.1 µL
DDH₂O	14.9 µL
Total	20 µL

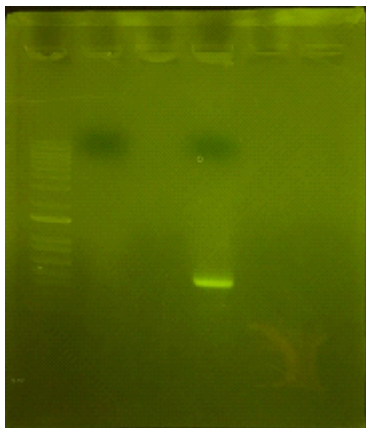
Target size: 542bp



correct

	SARS2- ORF7a T120I
Dream Taq Green Buffer	2 µL
Primer set	0.8 µL nCoV-ORF7a-F21
	0.8 µL nCoV-ORF8-R45
dNTP	0.4 µL
Template	1uL overgrown culture
Dream Taq	0.1 µL
DDH ₂ O	14.9 µL
Total	20 µL

Target size: 524bp

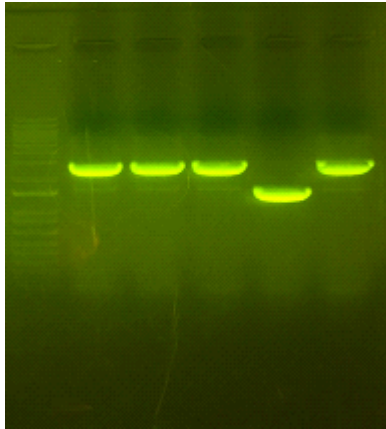


correct

	NSP12 G671S

Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L nCoV-NSP12-F1960
	0.8 μ L nCoV-NSP13-R604
dNTP	0.4 μ L
Template	1uL overnight grown culture
Dream Taq	0.1 μ L
DDH₂O	14.9 μ L
Total	20 μ L

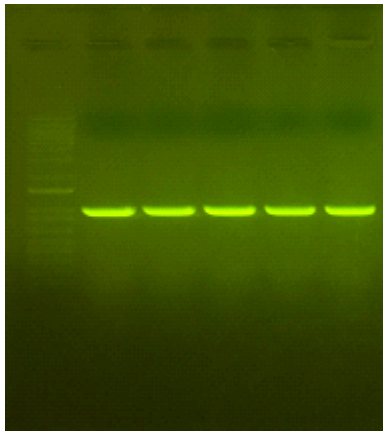
Target size: 1481bp



Colony 4 is correct, others are wrong

	N ENA del
Dream Taq Green Buffer	2 μ L
Primer set	0.8 μ L nCoV-orf7a-F359
	0.8 μ L nCoV-Nuc-R435
dNTP	0.4 μ L
Template	1uL overnight grown culture
Dream Taq	0.1 μ L
DDH₂O	14.9 μ L
Total	20 μ L

Target size: 969bp



All correct

PCR setting

		Temperature	Time
Initial denature		95 °C	3mins
Cycling (30 cycles)	Denature	95 °C	30s
	Annealing	55 °C	30s
	Extensiton	72 °C	1 min (2kb/1min, then 1kb/min)
Final Extension		72 °C	5mins
Store		4 °C	∞

Agarose Gel electrophoresis :

1. Prepare 25mL 1% agarose gel (0.25g agarose in 25mL 1X TAE), dissolve the agarose under the microwave
2. Cool the gel solution a little bit, then add 1.25μL (1:20000) SmartGlow to the gel solution and mix well
3. Pour the gel to the gel rack and wait for solidification.
4. Transfer the gel to the gel tank soaked with 1X TAE buffer
5. Add 10μL 6X DNA loading buffer to the samples (final conc. 1X), mixed well and loaded to the well of the gel, include one well loaded with DNA ladder for size referencing.
6. Run the gel at 120V (constant voltage) for 24mins.
7. Visualize the gel under Blue box

Midi-prep & Send sequencing

Midi-prep

1. Pour 250mL growth overnight culture in a 500mL centrifuge bottle (x2)
2. Centrifuge at 6000x g for 15mins at 4°C, remove supernatant
3. Resuspend the two pellets with 20mL **Resuspension Buffer (R3)** in one 250mL centrifuge bottle
4. Add 20mL **Lysis Buffer (L7)**, mix gently by inverting the bottle, incubate at room temperature for 5mins
5. Add 20mL **Precipitation Buffer (N3)**, mix immediately but gently by inverting the bottle
6. Centrifuge at ≥20,000 x g for 30 min at 4°C (16.250 rotor)
7. Place the MIDI column (with filtration cartridge) on the column holder with a tray to collect the flow through waste
8. Add 15mL **Equilibration buffer EQ1** to the column (10mL first, wait for flow through, then add another 5mL)
9. Pull all the supernatant after **step 6** to the MIDI column (with filtration cartridge) and wait for complete flow through
10. Remove the filtration cartridge from the MIDI column, add 10mL **Wash buffer (W8)** to the column and wait for complete flow through
11. Again, add 20mL **Wash buffer (W8)** to the column and wait for complete flow through
12. Add 3.5mL **99% Isopropanol** to the falcon
13. Place the column to the falcon, add 5mL **Elution buffer (E4)** to the column, wait for complete elute to the falcon
14. Mix the eluent with 99% Isopropanol well
15. Centrifuge at 6000x g for 1 hour at 4°C
16. Remove all supernatant, add 3mL **70% Ethanol** to resuspend the DNA pellet
17. Transfer the DNA pellet together with 70% Ethanol to a new 1.5mL eppendorf
18. Centrifuge at 16,000 g for 1min at room temperature

19. Remove all 70% Ethanol from the 1.5mL eppendorf
20. Repeat step 13-15 until all the DNA with 70% Ethanol is transferred from Falcon to the 1.5mL eppendorf
21. Dried the DNA pellet on 50°C heat block (around several seconds to 1min), be careful **NOT** completely dried the DNA pellet (completely transparent)
22. Add **~200µL pre-warmed 50°C ddH₂O** to dissolve the DNA pellet (warm on 55°C heat block with occasionally vortex)
23. Proceed to Nano Drop to measure the DNA concentration and purity, store this plasmid DNA at -20°C

Nsp13 P77L	1.275 ug/uL
Orf3a S26L	969.1 ng/uL
Orf7a T120I	975.4 ng/uL

Nsp12 G671S	926.5 ng/uL
N ERS del	2.52 ug/uL

Send sequencing

Send 250ng plasmid to Sanger Sequencing

Dilute plasmid 1:10

Set reaction:

	Nsp13 P77L	Orf3a S26L	Orf7a T120I
Primer	0.5	0.5	0.5
Plasmid	1.96	2.58	2.56
DDH2O	12.54	11.92	11.94
Total	15uL	15uL	15uL

	Nsp12 G671S	N ERS del
Primer	0.5	0.5
Plasmid	2.70	0.99
DDH20	11.80	13.51
Total	15uL	15uL

Sequencing Primer

SARS2-nsp13-P77L	nCoV-NSP12-F2741
SARS2-orf3a-S26L	nCoV-Spike-F3755
SARS2-orf7a-T120I	nCoV-ORF7a-F21

SARS2-nsp12-G671S	nCoV-NSP12-R2177
SARS2-N-ERSdel	nCoV-Orf8-F363

Transfection & Co-Culture

(1) Pass cell (BHK21 & VeroE6 TMPRSS2) in P2 Lab

21/2/2023

1. Use suction & glass pipette to remove all the medium in the plate
2. Add **1.5mL PBS** to wash the cell for twice (**VeroE6 TMPRSS2; BHK21 no need**)
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins for VeroE6 TMPRSS2; 2min for BHK21**
5. Add **6mL medium (with FBS)** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect to a 14mL centrifuge tube → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. At the same time, take a new plate, add **9mL medium** (with FBS) to it
9. After centrifugation, use suction and glass pipette to remove all the supernatant in the centrifuge tube
10. Add **4 mL (1:4)** and **7 mL (1:7)** medium to resuspend the pellet of VeroE6 TMPRSS2 and BHK21 respectively
11. Add **1mL** cell culture to the new plate

(2) Seed cell in 6-well plate (BHK21 & VeroE6 TMPRSS2) in P2 Lab

23/2/2023

1. Use suction & glass pipette to remove all the medium in the plate
2. Add **1.5mL PBS** to wash the cell for twice (**VeroE6 TMPRSS2; BHK21 no need**)
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins for VeroE6 TMPRSS2; 2min for BHK21**
5. Add **6mL medium (with FBS)** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect to a 50mL centrifuge tube → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 10mL DMEM medium and 10mL MEM respectively
10. Count cell

BHK21:

Target: 2×10^5 cells/mL

= 4×10^5 cells/well

= 4×10^5 cells/well X 8 well

= **3.2×10^6 cells**

Count: 68 --> $68 \times 4 / 4 = 68 \times 10^4 = 0.68 \times 10^6$ cells

= 3.2×10^6 cells / 0.68×10^6 cells

= **4.7mL cell + 11.3mL MEM+10%FBS**

VeroE6-TMP:

Target: 1.5×10^5 cells/mL

= 3×10^5 cells/well

= 3×10^5 cells/well X 8 well

= **2.8×10^6 cells**

Count: 72 --> $72 \times 4 / 4 = 72 \times 10^4 = 0.72 \times 10^6$ cells

= 2.8×10^6 cells / 0.72×10^6 cells

= **3.3mL cell + 12.7mL DMEM+10% FBS**

11. Add 2mL to each well of 6-well plate (Total: 7 well)

(3) Transfection & co-culture in P3 (SARS-CoV-2 WT Spike Point Mutation) in P3 Lab

24/2/2023

	Nsp12 G671S	Nsp13 P77L	Orf3a S26L	Orf7a T120I	N ENA del	+ve
DNA plasmid	10.8uL	6.89uL	11.3uL	11.3uL	9.89uL	19.3uL
Opti-MEM	240uL	240uL	240uL	240uL	240uL	230.7uL
Total	250uL	250uL	250uL	250uL	250uL	250uL

Opti-MEM	1547uL
Lipo2000	78uL (5X2.4X6.5)
Total	1625uL (6.5X)

12. Mixing the Opti-MEM – DNA and Opti-MEM – Lipofectamine 2000
Add 250µL Opti-MEM – Lipofectamine 2000 to each tube (1-6) and mixed well
13. Incubate at room temperature for 20 mins.
14. Add 500µL Opti-MEM – DNA – Lipofectamine 2000 mixture dropwise to well 1-6

After 6 hrs:

Re-seed the BHK-21 cell in plate 1 well 1-5 with DMEM (+1% FBS) at 6 hrs post-transfection

In P2: Aspirate all the medium in plate 2 well 1-5 (Vero-E6 cell), replace the medium with 1mL fresh **DMEM (+1% FBS)**

15. Aspirate all the medium in plate 1 well 1-6, add 1mL trypsin–EDTA solution and incubate at 37°C [5% CO₂] for ~2mins to detach the cell (slap the plate after 2mins)

16. Neutralize the trypsin–EDTA with **2mL DMEM (+10% FBS)**, transfer the transfected BHK-21 cell to a 1.5mL eppendorf
17. Centrifuge the cell at **450g for 7mins**
18. After the centrifugation, discard the supernatant, resuspend the BHK-21 cell pellet with **1mL DMEM (+1% FBS)**
19. Add the resuspended BHK-21 cell to plate 2 well 1-5 (Vero-E6 cell) and continue the incubation at 37°C, 5% CO₂ [~72hrs]

(4) Seed cell in 6-well plate (VeroE6 TMPRSS2) in P2 Lab

27/2/2023

1. Use suction & glass pipette to remove all the medium in the plate
2. Add **1.5mL PBS** to wash the cell for twice
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins**
5. Add **6mL medium (with FBS)** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect to a 50mL centrifuge tube → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 13mL DMEM medium
10. Add 2mL to each well of 6-well plate (Total: 6 well)

(5) Pass viral supernatant to fresh VeroE6-TMPRSS2 in P3 Lab

28/2/2023

1. Transfer 2mL co-culture medium to 1.5mL eppendorf
2. Centrifuge for 5 min at MAX speed
3. Remove all medium from fresh VeroE6-TMPRSS2
4. Wash with 1mL PBS
5. Replace with the viral supernatant to the fresh cell
6. Incubate at 37°C for 2 days

Repeat step 3,4,5 for other 5 wells (handle the MOCK first "the most cleanest")

(6) Collect P0 Viral stock in P3 Lab

2/3/2023

1. Transfer viral supernatant to 1.5mL eppendorf
2. Centrifuge for 5 min at MAX speed
3. Aliquot viral supernatant into two 1.5mL screw cap, 350uL each
(For each virus)

Plaque Assay

(1) Seed cell in P2 Lab

13/3/2023

1. Use suction & glass pipette to remove all the medium in the plate (5X10cm dish)
2. Add **1.5mL PBS** to wash the cell for twice
3. Remove the PBS
4. Add **1mL Trypsin** and incubate for **4 mins**
5. Add **6mL medium (with FBS)** to the plate (neutralize the trypsin, otherwise enzyme will cut the cell continuously)
6. Collect into 2X 50mL centrifuge tubes → pipette up & down
7. Centrifuge for **5mins**, room temp, 1000 rpm
8. Remove supernatant
9. Resuspend the cell pellet with 32mL DMEM medium X2
10. Add 2mL to each well of 6-well plate (Total: 5 plate)

(2) Plaque Assay in P3 Lab

14/3/2023

1. Add **135uL** medium in each well (6 well in 96-well plate)
2. Remove medium from the clean cell
3. Add **1mL plain medium to clean cell (6-well)**
4. Transfer **15uL** viral supernatant into 1st well of 96-well plate
5. --> Pipete up and down
6. Transfer 15uL culture from 1st well to 2nd well --> repeat until the 6th well
7. Add **100uL serial diluted medium to clean cell** (from low conc. to high conc.)

8. Incubate for 1 hr
9. Prepare **50ml 2XDMEM with 2% FBS** --> warm in 37°C water bath
10. Prepare **50mL 1.8% low-melting agarose** --> warm in 37°C water bath
11. Pour 50ml 2XDMEM with 2% FBS into 50mL agarose --> shake
12. Remove all the medium from cell (from low conc. to high conc.)
13. Add 3mL agarose with medium to each well (6-well)
14. --> wait for 5 min
15. Stick tape
16. Incubate for 3 days (72hrs)

(3) Fix Plaque in P3 Lab

17/3/2023

Prepare 120mL 4% PFA in 1X PBS in P2 Lab

[30mL 40% PFA in 90mL 1XPBS (Bench)]

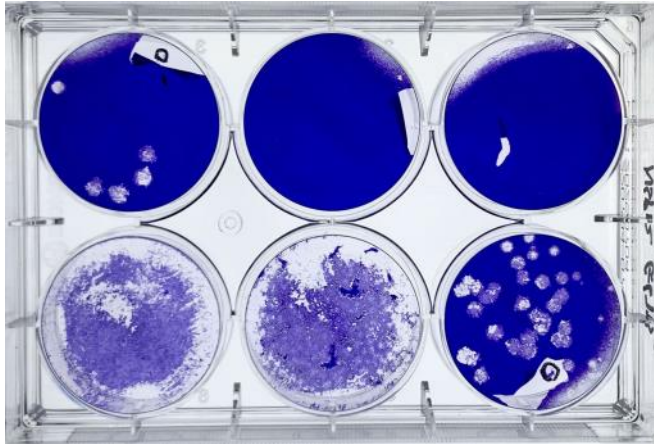
1. Add 3mL 4% PFA into each well
2. Place in room temp overnight

(4) Stain Plaque in P3 Lab

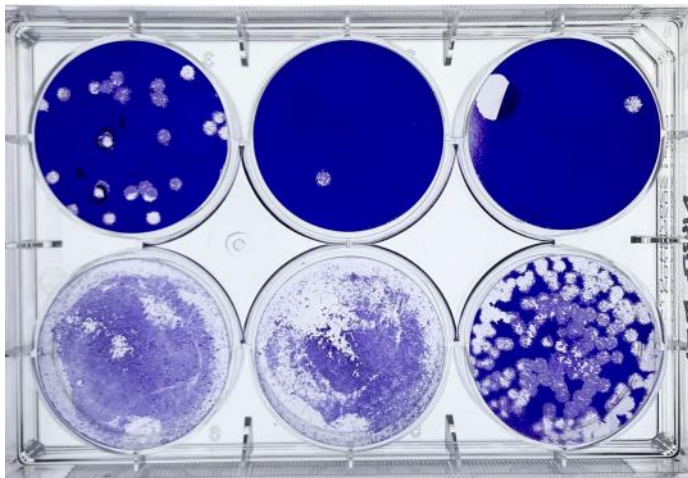
20/3/2023

1. Remove the agar
2. Immerse in Virkon
3. Wash with tap water
4. Stain with Crystal violet (0.5% crystal violet in 10% Ethanol)
5. Remove crystal violet after 10min
6. Wash with tap water

Nsp12 G671S



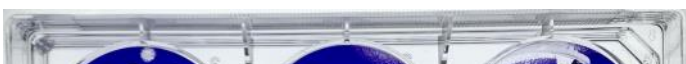
Nsp13 P77L

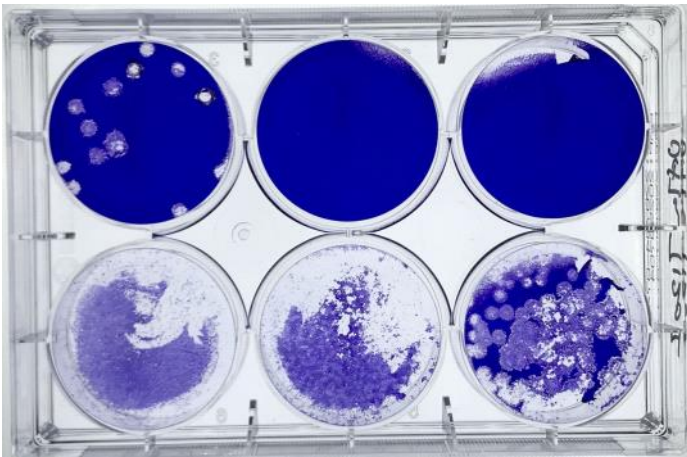


Orf3a S26L



Orf7a T120I





N ERS del

