

**OPERATIONAL SUPPORT**



**Update of standard laboratory  
protocols for SARS-CoV-2  
characterisation**

**2026**

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Any commercial services/kits that are mentioned in the handbook during specific protocols, are only meant for illustrative purposes. Similar services/kits are available from other commercial entities.

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## Abbreviations

ACE2	Angiotensin-converting enzyme 2
AURORAE	Laboratory sUpport fOr influenza and coRonA for Europe
ARDS	Acute respiratory distress syndrome
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BSL-3	Biosafety level 3
CC	Cell control
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CME	Clathrin-mediated endocytosis
COVID-19	Coronavirus disease 2019
CPE	Cytopathic effect
dpi	Days post infection
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
E	Envelope protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EVAg	European virus archive - GLOBAL
FB	Flush buffer
FBS	Fetal bovine serum
FLT	Flush tether
FRNT	Focus reduction neutralisation test
FVMM	Fast virus master mix
g	G force
GFP	Green fluorescent protein
HCS	High-content screening
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
Ig	Immunoglobulin
IgM	Immunoglobulin M
IMDM	Iscove's modified Dulbecco's medium
IU	International unit
LB	Loading Beads
LLOQ	Lower limit of quantification
M	Membrane protein
MEM	Minimum essential medium
MOI	Multiplicity of infection
MWCO	Molecular weight cut-off
N	Nucleocapsid
NaHOC3	Sodium hydrogen carbonate
NC	Negative control
NEAA	Non-essential amino acids
NGS	Next generation sequencing
OD	Optical density
PBS	Phosphate buffered saline
PC	Positive control
Pen/Strep	Penicillin-Streptomycin
PES	Polyethersulfone
PFU	Plaque-forming unit
PhHV	Phocine herpes virus
PP	Pseudoparticles
PRNT	Plaque reduction neutralisation test
PRNT <sub>50</sub>	Plaque reduction neutralisation test with 50% reduction
qRT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RBD	Receptor-binding domain
RFA	RNase free water
RNA	Ribonucleic acid
ROI	Region of interest

rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
S	Spike protein
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SC	Serum control
SFB	Short fragment buffer
SQB	Sequencing buffer
PIV5	Parainfluenza virus 5 (previously known as simian virus 5)
TCID <sub>50</sub>	Tissue culture infectious dose
TFA	4x TaqMan Fast Advanced Master Mix with UDG
TMPRSS2	Transmembrane protease, serine 2
UDG	Uracil-DNA glycosylases
VSV-g	Vesicular stomatitis virus G glycoprotein
VC	Virus control
VNT	Virus neutralisation test
VOC	Variant of concern
WHO IS	World Health Organisation international standards
w/v	Weight by volume

# Executive summary

This handbook provides an updated overview of commonly used laboratory protocols for the characterisation of SARS-CoV-2, provided by the AURORAE consortium. Basic cell culture protocols are presented, as well as specific protocols for testing SARS-CoV-2 and influenza-positive, or clinical specimens. The protocols include methods such as plaque assays, virus titration, antibody-based detection methods, virus neutralisation assays, as well as protocols to perform sequencing and PCRs. This handbook also highlights the variability between protocols used in different institutes, and points out different ways of performing the assays at appropriate points. However, it is not possible to show the entirety of this variability within the scope of this handbook. Furthermore, an adaptation of the protocols may be necessary upon establishment.

Updates in this report include:

- Replacement of the general cell culture protocol (2.1). Updated version now also includes cultivation advice for VeroE6 TMPRSS cells.
- Replacement of the amplicon sequencing on the MinIon (2.5.1).
- Replacement of the virus neutralisation test for SARS-CoV-2 (2.6.3). The updated version contains minor changes.
- Added a protocol for virus neutralisation test for SARS-CoV-2 with staining (2.6.4).

## 1 Background

### 1.1 General background

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) resulted in the worldwide pandemic of coronavirus disease 2019 (COVID-19), which resulted in over 778 million confirmed cases worldwide, and over 7.1 million deaths (as of 11 December 2025) [1].

### 1.2 Current state of knowledge

The coronavirus virion consists of the proteins: nucleocapsid (N), membrane (M), envelope (E), and spike (S) [2]. The latter is composed of two subunits, S1 and S2, showing a crown-like formation on the surface of the virion. Subunit S1 contains the receptor-binding domain (RBD), whereas subunit S2 is involved in the membrane fusion between virus and cell [3].

To enter cells, SARS-CoV-2 relies on the angiotensin-converting enzyme 2 (ACE2) [2], which is expressed in many organs, including the bronchi, lung parenchyma, heart, kidney, and gastrointestinal tract [3, 4]. Binding of SARS-CoV-2 to ACE2 induces conformational changes in subunit S1 and exposes the cleavage site S2' in subunit S2. Thereafter, the S2' site is cleaved by different proteases, depending on the entry route taken by the virus. If transmembrane protease serine 2 (TMPRSS) is lacking, the virus enters the cell via clathrin-mediated endocytosis (CME). To permit membrane fusion, S2' is cleaved by cathepsins. In the presence of TMPRSS2, S2' is cleaved at the cell surface. In both cases, cleavage of the S2' site exposes the fusion peptide. Furthermore, conformational changes are induced by the dissociation of S1 from S2. Membrane fusion is then initiated by forwarding the fusion peptide into the target membrane. A fusion pore is formed leading to the fusion of viral and cellular membranes, through which viral ribonucleic acid (RNA) is released into the host cell cytoplasm [2].

Although most individuals suffering from SARS-CoV-2 infection develop only mild to moderate illness characterised by a replication of the virus in the upper respiratory tract, some develop life-threatening pneumonia after infection [5]. In severe cases, infection with SARS-CoV-2 can lead to an over-activation of the host's immune system, resulting in an excessive inflammatory response associated with the increased release of cytokines (termed cytokine storm), which is commonly seen in acute respiratory distress syndrome (ARDS), sepsis, and fulminant multiorgan failure [3].

Various mutants have emerged from the ancestral strain, resulting in a constant circulation of SARS-CoV-2 variants [6]. Even though coronaviruses exhibit a proofreading function that reduces their error rate compared to other RNA viruses, accumulations of mutations still occur in the viral genome. While some of the mutations are synonymous, others lead to a change in the amino acid sequence or truncations that have an impact on infection and spread [7]. Mutations in the S protein are associated with modified intrinsic virus properties and immune escape [6].

The antigenic distance between variants can be visualised and quantified using antigenic maps, where antiserum and antigens are positioned on a grid based on corresponding neutralising titres. Continuous usage of this method and including new variants in the maps can help to improve the understanding of the evolution of SARS-CoV-2 [8] and predict the optimal spike antigen to include in vaccine formulations in the future.

## 1.3 Purpose

Genetic and antigenic characterisation of the virus is essential to study SARS-CoV-2, and identify new mutations or investigate the interaction of new virus variants and patient sera or standard sera. This handbook presents assays for virus detection by reverse transcription-polymerase chain reaction (RT-PCR), isolation of the virus, sequencing, as well as antibody-based assays. Without the availability of a biosafety level-3 (BSL-3) laboratory, or if full viruses are not available pseudovirus particles can be used to study the effect of spike mutations. We have also included these protocols in this handbook.

All the methods for testing SARS-CoV-2 positive-samples were developed during the pandemic, starting with the detection of this novel coronavirus by real-time PCR [9]. Meanwhile, for some assays, such as antibody-based detection methods, kits are commercially available. We have indicated this information in pertinent sections in the handbook.

## 2 Methods

Possible manufacturers are indicated for the reagents used in the protocols presented here. Reagents from other manufacturers can also be used if an equivalent quality is guaranteed. If necessary, a validation of the protocols must take place.

For the centrifugation steps, the centrifugation speed can be converted from relative centrifugal force (RCF) or g force (g) to revolutions per minute (rpm), or vice versa with a reliable unit conversion tool.

### 2.1 Cell culture

#### 2.1.1 Biosafety

The implementation of the protocols listed in this handbook require different laboratory safety levels.

Cultivation of uninfected cells can take place under BSL-1 conditions [10].

The execution of protocols with pseudoviruses requires a security level of BSL-2 [11].

At the time of compiling this handbook, SARS-CoV-2 is classified as risk group 3 according to the EU Directive 2000/54/EC [10]. It is to be cultivated under BSL-3 conditions, according to the national regulations and regulatory authorities.

#### 2.1.2 Cultivation of cells

##### *Introduction*

In this section, the cultivation of different cell cultures is presented. These are required for the implementation of further protocols listed in this handbook. The specific cells used for specific assays are noted at pertinent places.

Cells should routinely be tested for mycoplasma and parainfluenza virus 5 (PIV5) (see chapter 2.1.2 and 2.1.3). The composition of the media may vary depending on the laboratory. Therefore, several options are provided here. Once a cultivation medium has been chosen, it should ideally not be changed.

The fetal bovine serum (FBS) used for the medium must be heat-inactivated for 30 minutes at 56 °C before use.

##### *Materials and reagents*

- pipettes;
- PIPETBOY;
- filter tips;
- serological pipettes;
- falcon tubes;
- flasks;
- medium;
- cells.

### **Calu-3 cells**

ATCC number: HTB-55 [12]

It is recommended to keep the cells sub-confluent. Therefore, a sub-cultivation ratio of 1:3 to 1:6 is ideal.

Comment: When plates are seeded for an experiment, the wells must of course be confluent. For this purpose,  $6 \times 10^5$  cells/mL (=  $3 \times 10^5$  cell per well for a 24-well plate) can be seeded. Wells are confluent the next day.

The following is an overview of the different culture media used:

#### **Culturing medium**

Opti-MEM + GlutaMAX (without NaHCO<sub>3</sub> HEPES; Gibco, 51985-026), supplemented with:

- 10% FBS (Sigma-Aldrich, F7524);
- 1x Penicillin/Streptomycin (Pen/Strep) (10 000 U/mL; 10 000 µg/mL; Capricorn Scientific, CA PC-B or Lonza, 17-602E);
- 0.0005 mg/mL Amphotericin B (0.25 mg/mL; from pharmacy).

Comment: Cultivation is also possible using DMEM (with high glucose, L-Glutamine, without sodium pyruvate; Gibco, 41965039) supplemented with:

- 10% FBS (Thermo Fisher Scientific, 10270106);
- 2 mM L-Glutamine (200 mM; Life Technologies, 15630056);
- 1x PenStrep (10 000 U/mL; 10 000 µg/mL; Lonza, 17-602E).

For infections, the same medium with a reduced amount of FBS (2%) can be used.

#### **Infection medium**

Advanced DMEM/F-12 (without NaHCO<sub>3</sub> HEPES, L-Glutamine; Gibco, 12634010) supplemented with:

- 10 mM HEPES (1 M; Lonza, 17-737E);
- 2 mM GlutaMAX (200 mM; Gibco, 35050-038);
- 1x Pen/Strep (10 000 U/mL; 10 000 µg/mL; Capricorn Scientific, CA PC-B or Lonza, 17-602E) ;
- 0.0025 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

### **Vero-118 cells**

A cultivation ratio of 1:4 to 1:10 is recommended.

Comment: When plates are seeded for an experiment,  $3 \times 10^5$  cells/mL (=  $1.5 \times 10^5$  cells per well for a 24-well plate) can be seeded. Wells are confluent the next day.

The following is an overview of the different culture media used:

#### **Culturing medium**

IMDM (Capricorn Scientific, CP22-5020) supplemented with:

- 10% FBS (Sigma-Aldrich, F7524);
- 4 mM L-Glutamine (200 mM; Life Technologies, 15630056);
- 1x Pen/Strep (10 000 U/mL; 10 000 µg/mL; Capricorn Scientific, CA PC-B) ;
- 0.0005 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

#### **Infection medium**

IMDM (Capricorn Scientific, CP22-5020) supplemented with:

- 10% FBS (Sigma-Aldrich, F7524);
- 4 mM L-Glutamine (200 mM; Life Technologies, 15630056);
- 1x Pen/Strep (10 000 U/mL; 10 000 µg/mL; Capricorn Scientific, CA PC-B) ;
- 0.0025 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

***Ver0-E6 cells***

ATCC number: CRL-1586 [13]

A sub-cultivation ratio of 1:4 to 1:10 is recommended.

Comment: When plates are seeded for an experiment,  $3 \times 10^5$  cells/mL ( $=1.5 \times 10^5 \times 2$  cells per well for a 24-well plate) can be seeded. Wells are confluent the next day.

The following is an overview of the different culture media used:

**Culturing medium**

DMEM (without HEPES and L-Glutamine, Capricorn Scientific, DMEM-HPXA) supplemented with:

- 10% FBS (Sigma-Aldrich, F7524);
- 4 mM L-Glutamine (200 mM; Life Technologies, 15630056);
- 1x Pen/Strep (10 000 U/mL; 10 000 µg/mL; Capricorn Scientific, CA PC-B) ;
- 0.0005 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

Comment: Cultivation is also possible using DMEM (with high glucose, L-Glutamine, without sodium pyruvate; Gibco, 41965039) supplemented with:

- 10% FBS (Thermo Fisher Scientific, 10270106);
- 1% NEAA (Gibco, 11140035);
- 1% sodium pyruvate (Gibco, 11360039).

**Infection medium**

DMEM (without HEPES and L-Glutamine, Capricorn Scientific, DMEM-HPXA) supplemented with:

- 20 mM HEPES (1 M; Life Technologies, 25080102);
- 10% FBS (Sigma-Aldrich, F7524);
- 4 mM L-Glutamine (200 mM; Life Technologies, 15630056);
- 1x Pen/Strep (10 000 U/mL, 10 000 µg/mL; Capricorn Scientific, CA PC-B);
- 0.0005 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

***Ver0E6 TMRSS cells***

A subcultivation ratio of 1:4 – 1:10 is recommended.

Comment: when plates are seeded for an experiment  $3 \times 10^5$  cells/mL ( $=1.5 \times 10^5 \times 2$  cells per well for a 24 well plate) can be seeded, wells are confluent the next day.

Overview of the different culture media used for different purposes:

**Culturing medium:**

DMEM (without HEPES and L-Glutamine, Capricorn, DMEM-HPXA) supplemented with:

- 10% FBS (Sigma, F7524);
- 4 mM L-Glutamine (200 mM; Life Technologies, 15630056,);
- 1x PenStrep (10,000 U/mL; 10,000 µg/mL; Capricorn, CA PC-B);
- 0.0005 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

Comment: cultivation is also possible using DMEM (with high glucose, L-Glutamine, without sodium pyruvate; Gibco, 41965039) supplemented with:

- 10% FBS (ThermoScientific, 10270106);
- 1% NEAA (Gibco, 11140035);
- 1% sodium pyruvate (Gibco, 11360039).

**Infection medium:**

DMEM (without HEPES and L-Glutamine, Capricorn, DMEM-HPXA) supplemented with:

- 20 mM HEPES (1 M; Life Technologies, 25080102);
- 10% FBS (Sigma, F7524);
- 4 mM L-Glutamine (200 mM; Life Technologies, 15630056,);
- 1x Pen/Strep (10,000 U/mL, 10,000 µg/mL; Capricorn, CA PC-B);
- 0.0005 mg/mL Amphotericin B (0.25 mg/mL; Pharmacy).

**HEK-293T**

ATCC number: CRL-3216 [14]

A sub-cultivation ratio of 1:3 to 1:8 is recommended.

**Culturing medium**

DMEM (Gibco) supplemented with:

- 10% FBS (Sigma-Aldrich, F7524);
- 1x Pen/Strep (10 000 U/mL; 10 000 µg/mL; Lonza, 17-602E);
- 1x NEAA.

**2.1.3 Mycoplasma testing**

Testing for mycoplasma is offered by several companies, such as Eurofins Scientific.

Sample preparation:

- Collect 500 µL cell culture supernatant;
- Incubate for 10 minutes at 90°C;
- Centrifuge at 13 000 rpm for 10 seconds;
- Transfer 100 µL into a new reaction vessel.

The sample is barcoded and sent to the company, where the extraction and PCR-based testing for mycoplasma takes place.

**2.1.4 In-house mycoplasma testing**

A PCR-based protocol can be used for in-house mycoplasma testing.

**Method****Nucleic acid extraction**

- Extract total nucleic acids from 500 µL supernatant, spiked with a known concentration of Phocine herpesvirus (PhHV) as internal control.
- Kits and protocols:
  - MagNa Pure 96 DNA kit (Roche);
  - MagNa Pure 96 Viral NA LV kit (Roche);
  - Viral NA Universal LV 2.0 protocol.
- Resuspend nucleic acids in a final volume of 100 µL.
- Include positive and negative controls in the whole process.

**Internal control detection****PCR**

The PCR is performed in a Roche LightCycler 480 Instrument-II with the following thermocycling protocol:

Five minutes at 50 °C, 20 seconds at 95 °C, 45 cycles (three seconds at 95 °C, 30 seconds at 60 °C).

**PCR mix**

Reagent	Volume
4x TaqMan Fast Advanced Master Mix with UDG*	5.0 µL
PhHV primers and probe mixture (see Table 1)	0.4 µL
PCR-grade Water	6.6 µL
Eluate	8.0 µL
<b>Total</b>	<b>20.0 µL</b>

\*TFA, Thermo Fisher Scientific Inc.,

**Mycoplasma species detection****PCR**

PCR is performed in a Roche LightCycler 480 Instrument-II with the following thermocycling protocol:

15 minutes at 95 °C, 40 cycles [one minute at 95 °C, one minute at 57 °C, and one minute at 72 °C], 10 minutes at 72 °C.

**PCR mix**

Amplify eluate using the HotStarTaq DNA polymerase kit (QIAGEN)

Reagent	Volume
10 x PCR buffer	5.0 µL
MgCl <sub>2</sub> (25 mM)	2.0 µL
dNTP (10 mM)	1.0 µL
HotStarTaq DNA polymerase	0.5 µL
Primers (see Table 2)	1.0 µL
Eluate	10.0 µL
PCR-grade Water	29.5 µL
<b>Total</b>	<b>50.0 µL</b>

**Detection of PCR products**

- Detect PCR products (282 bp) by gel electrophoresis on a 2% agarose gel;
- Samples are considered positive if a clear band is detected at the same height as the positive control;
- Samples are considered negative if no band is detected on the agarose gel and the internal control value is in range;
- Samples which are negative with an out-of-range internal control, will be repeated.

**Table 1. Primers and probes for internal controls of the in-house mycoplasma testing**

Oligo name	Sequence 5'→3'	pmol/reaction
PhHV-1-fwd	GGGCGAATCACAGATTGAATC	2.5
PhHV-1-rev	GCGGTTCCAACGTACCAA	10
PhHV-1-probe	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2	5

Primers and probes are adapted from Doornum et al [15].

**Table 2. Primers for in-house mycoplasma testing**

Oligo name	Sequence 5'→3'	pmol/reaction
MSP-fwd	GGGAGCAAACAGGATTAGATACCCT	20
MSP-rev	TGCACCATCTGTCACTCTGTTAACCTC	20
MSP-rev2	TGCACCACCTGTCACTGGGTTGACCTC	20

Primers are adapted from Kuppeveld et al [16].

**2.1.5 In-house parainfluenza virus 5 (PIV5) testing**

Testing for PIV5 is performed by PCR.

**Materials and reagents**

- pipettes;
- filter tips;
- reaction vessels;
- viral RNA extraction kit, e.g. from MACHEREY-NAGEL.

**Method**

- Collect 75 µL cell culture supernatant;
- Extract RNA using a viral RNA extraction kit, according to the manufacturer's instructions;
- Perform PCR as follows:

**PCR mix**

Reagent	Volume
RFA	1.5 µL
2rxn buffer	6.25 µL
BSA	0.5 µL
SSIII/PlatTaq-Pol. [5 U/µl]	0.5 µL
F-Primer [10 µM]	0.5 µL
R-Primer [10 µM]	0.5 µL
Probe [10 µM]	0.25 µL
RNA	2.5 µL
<b>Total</b>	<b>12.5 µL</b>

RT-PCR is done on a Roche LightCycler 480 Instrument-II, with the following thermocycling protocol: 20 minutes at 55 °C, three minutes at 94 °C, 45 cycles [15 seconds at 94 °C, 30 seconds at 58 °C].

### Primer-probe (PP-)mixes

**Table 3. Primers and probes for in-house PIV5 testing**

Primer/Probe	Sequence	Fluorochrome
PIV5-F (BN)	CGT GGG GGA TCC CTT CA	
PIV5-P (BN)	CCCACCAGCAGATACCAG	5'-/56-FAM/CCC ACC AGC /ZEN/AGA TAC CAG TCA ATT TGA TC/3IABkFQ/-3'
PIV5-R (BN)	CCA CCT CTG GGT GAT ACA ATG A	

## 2.1.6 Freezing of cells

### Introduction

Permanent preservation of vital eukaryotic cells is performed in liquid nitrogen.

### Materials and reagents

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- falcon tubes;
- cryotubes;
- freezing container;
- PBS;
- trypsin-EDTA (0.05% or 0.25%) (Gibco, 25300062 or 25200056);
- culture medium without additives;
- freezing medium: 80% FBS + 20% DMSO.

### Method

- Approximately,  $1 \times 10^7$  cells/cryotube are frozen;
- Discard medium and wash cells with PBS once;
- Cells are trypsinised with 0.05% or 0.25% trypsin-EDTA for 3–15 minutes, depending on the cell line;
- Cells are pelleted by spinning down (1 400 rpm, five minutes, RT);
- Cell pellet is resuspended in 0.5 mL of culture medium without further additives;
- Resuspended cells are placed in cryotubes with an equal volume of freezing medium;
- Cells are frozen at -80°C using a freezing container. Thereafter cells are transferred to liquid nitrogen vapour phase.

## 2.1.7 Thawing of cells

### Introduction

Work fast while thawing cells to wash out the DMSO contained in the freezing medium as quickly as possible.

After thawing, the cells are passaged twice before use to ensure consistent conditions.

### Materials and reagents

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- falcon tubes;
- culture medium;
- flasks.

### Method

- Falcon tubes are prepared with 10 mL culture medium;
- Cells are thawed;
- Cells are either thawed in a water bath at 37 °C, by heating in the hand, or by using a warmed tube of ethanol at 37 °C;
- Thawed cells are transferred directly in prepared medium;
- Cells are pelleted by centrifugation (1 400 rpm, five minutes, RT);
- Supernatant is discarded;

- Cells are washed with culture medium;
- Cells are cultured according to standard methods.

## 2.2 Generation of viral stocks

### 2.2.1 Virus culture from clinical PCR-positive specimens

#### *Introduction*

SARS-CoV-2 is isolated from positive clinical specimens for various purposes. Vero-E6 cells (seed  $3 \times 10^5$  cells/mL, one or two days in advance) can be used for most isolates. Depending on the experiment, it may be also useful to use Calu-3, Vero-hSLAM or Vero-TMPRSS. Usage of Vero-TMPRSS can increase the number of infected cells and the amount of viral RNA in the cell culture supernatant [17]. The usage of Vero-hSLAM for serial propagation can improve the overall genetic stability of working stocks [18].

#### *Materials and reagents*

- PIPETBOY
- serological pipettes;
- pipettes;
- filter tips;
- falcon tubes;
- reaction vessels;
- PBS;
- cells in a 24-well plate;
- CO<sub>2</sub> incubator
- fluid from PCR-positive swab.

#### *Method*

Fluid from a PCR-positive swab from the anterior nose or the nasopharynx is filtered through a 24 µm filter.

##### **Passage 1**

- Wash monolayer of cells in a 24-well plate with PBS.
- Add 1 mL medium to the cells.
- Add 200 µL filtered swab fluid to the cells.
- Centrifuge the plate for 10 minutes at 350xg.
- Incubate the plate at 37 °C in a CO<sub>2</sub> incubator.
- Check the cells daily for CPE.
- Harvest the supernatant when 50–75% of the cells show CPE.

##### **Passage 2**

- Wash monolayer of cells in a T25 cell culture flask with PBS.
- Add the supernatant from Passage 1 to 10 mL medium.
- Incubate at 37 °C in a CO<sub>2</sub> incubator.
- Check the cells daily for CPE.
- Harvest the supernatant when 50–75% of the cells show CPE.

##### **Passage 3**

- Wash monolayer of cells in a T175 cell culture flask with PBS.
- Add the supernatant from Passage 2 to 40 mL medium.
- Incubate at 37 °C in a CO<sub>2</sub> incubator.
- Check the cells daily for CPE.
- Harvest the supernatant when 50–75% of the cells show CPE.
- Centrifuge the supernatant at 1 000xg for 10 minutes.
- Transfer the supernatant to a fresh tube.
- Centrifuge the supernatant again at 1 000xg for 10 minutes.
- Aliquot and store at -80 °C.

Depending on further use, TCID<sub>50</sub> or focus/plaque-forming units are determined.

The propagated virus stock is sequenced to warrant its integrity. Virus stocks that are made available via the [European virus archive – GLOBAL \(EVAg\)](#), the [Medicines and Healthcare products Regulatory Agency \(MHRA\)](#) or other repositories are quantified by qRT-PCR, sequenced to confirm the virus variant and the absence of virus-compromising mutations, and tested to confirm the absence of mycoplasma or PIV5. Attention should also be paid to other contaminations that may occur in a cell culture. For this, genomes of typical contaminations can be checked during sequencing.

## 2.2.2 Generation of SARS-CoV-2 stock from pre-existing stocks

### Introduction

SARS-CoV-2 is isolated from positive clinical specimens for different purposes. Vero-E6 cells (seed  $3 \times 10^5$  cells/mL one or two days in advance) can be used for most isolates. Depending on the experiment, it may also be useful to use Calu-3, Vero-hSLAM, or Vero-TMPRSS. Usage of Vero-TMPRSS can increase the number of infected cells and the amount of viral RNA in the cell culture supernatant [17]. The usage of Vero-hSLAM for serial propagation can improve the overall genetic stability of working stocks [18].

### Materials and reagents

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- falcon tubes;
- cryotubes;
- Vivaspin columns (Sartorius Stedim Biotech, Vivaspin 20 VS 2041, 100 000 MWCO polyethersulfone);
- PBS;
- cells;
- virus supernatant;
- infection medium: DMEM with 2% FBS, 1% NEAA, 1% sodium pyruvate;
- gelatine medium: 5% gelatine (Fluka 48723-500G-F) is dissolved in water (5 g in 100 mL distilled water) and autoclaved.
  - Dilute 1:10 in OptiPRO.
  - Dilute samples 1:2 in gelatine medium.

### Method

#### Stock production

- Vero-E6 cells (cultivation according to protocol) are seeded in T175 flasks the day before.
- The cells should be 90% confluent the next day.
- The medium is discarded and the cells are washed once with 10 mL PBS.
- The cells are infected with virus stock.

Comment: As a rule of thumb, 20 mL infection medium with 100  $\mu$ L virus stock is used for infection. If the titre of the virus stock is known, a defined MOI (MOI of 0.00025 to 0.0005) can be used for infection.

- The infected cells are cultured for three days at 37 °C, and 5% CO<sub>2</sub>.

Comment: The incubation time may vary from virus to virus.

- After ~3 dpi, 80–90% of the cells should be dead. Otherwise, cells are cultivated one day longer to harvest.

Comment: The supernatant can be harvested when CPE is visible.

- The supernatant is harvested and transferred to a 50 mL tube.

#### Vivaspin purification

- The transferred supernatant is centrifuged for 5–10 minutes, at 3 000xg.
- Vivaspin columns are equilibrated by pipetting 10 mL PBS up and down the column several times, followed by centrifuging for 10 minutes at 3 000xg.
- 20 mL of supernatant is added to the Vivaspin columns and then centrifuged for approximately one hour at 4 000xg.

Comment: After centrifugation, a maximum of 0.5 mL supernatant should be left above the filter; 200  $\mu$ L is better. The centrifugation time may vary, depending on the virus used.

- The flow through is discarded.
- Add 3 mL PBS to the virus concentrate and pipette up and down.
- Add 3 mL gelatine medium to the virus concentrate.
- Aliquot the virus dilution into cryotubes (e.g. 250  $\mu$ L aliquots).
- Freeze at -80 °C.

### Quality check virus stock

- Infectious titre determination: Titrate thrice independently (use different aliquots, at least on two different days, and at least two different cell passages).

Comment: The titre is usually between 1–3 000 000 PFU/mL.

Determination of genome equivalents: Take a sample for the extraction of viral RNA.

For example: For viral RNA extraction, use 50 µL viral stock and 300 µL lysis buffer RAV1 from MACHEREY-NAGEL, or 50 µL viral stock and external lysis buffer from QIAGEN. The extraction is followed by SARS-CoV-2 E-gene assay. The CDC N-gene assay can also be used.

The qRT-PCR assay can be calibrated with the WHO IS (currently 20/146, available on the [NIBSC website](#)).

- Perform next generation sequencing (NGS) and check for mutations.

## 2.3 Virus titration

### 2.3.1 TCID<sub>50</sub>

#### Introduction

This protocol describes the method to determine the TCID<sub>50</sub> of SARS-CoV-2.

SARS-CoV-2 is isolated from positive clinical specimens for various purposes. Vero-E6 cells (seed 3x10<sup>5</sup> cells/mL, one or two days in advance) can be used for most isolates. Depending on the experiment, it may be also useful to use Calu-3, Vero-hSLAM, or Vero-TMPRSS. Usage of Vero-TMPRSS can increase the number of infected cells and the amount of viral RNA in the cell culture supernatant [17]. The usage of Vero-hSLAM for serial propagation can improve the overall genetic stability of working stocks [18].

#### Materials and reagents

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- 96-well flat-bottom plates;
- 96-well U-bottom plates;
- Vero-E6 cells;
- Calu-3 cells;
- culture medium for Vero-E6 cells;
- culture medium for Calu-3 cells;
- SARS-CoV-2 virus stock.

#### Method

##### Preparation of cell plates

- Prepare cells ahead of time, depending on the cell line used.
- Prepare 96-well flat-bottom plates.
- Seed 50 000 Vero-E6 cells (medium), or 40 000 Calu-3 cells (medium) per well in a 96-well plate.

##### Titration

- Add 180 µL medium to each well (U-bottom plates).
- Add 20 µL virus to wells in column 1.
- Dilute from left to right (columns 1–10), by transferring 20 µL to the next column until column 10.
- Mix carefully and change filter pipette tips in between.
- Columns 11–12 serve as negative controls; no virus is added.
- Transfer 100 µL of each well to the Vero-E6/Calu-3 flat-bottom plates.
- Incubate at 37 °C for 5–7 days.
- Score based on the CPE, and calculate the TCID<sub>50</sub> based on the Spearman–Kärber formula [19].

## 2.3.2 Titrating stock for PRNT<sub>50</sub> using the Cellular Technology Limited (CTL) ImmunoSpot analyser

### Introduction

This protocol describes the method to determine the working dilution of SARS-CoV-2 by calculating the plaque-forming units (PFU) per mL using the CTL ImmunoSpot analyser. Other protocols can also be used for titration of virus stocks, e.g. using crystal violet instead of an antibody-based detection, and counting plaque number by eye (see section 2.6.1 for comparison).

Vero-E6 can be used for pre-VOC isolates. For VOC variants, Calu-3 cells should be used as Vero cells are less permissive for these variants.

### Materials and reagents

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- 96-well flat-bottom plates;
- 10% formalin;
- ethanol;
- PBS;
- 0.5% Triton X-100-PBS;
- blocking buffer: 20% w/v (weight by volume) skimmed milk in PBS;
- primary antibody: SARS-CoV-2 rabbit polyclonal antibody (Catalog#40143-T62, Sino Biological); 1:1 000, diluted in blocking buffer;
- secondary antibody: polyclonal goat anti-rabbit immunoglobulins/HRP (Cat#P044801-2, Agilent); 1:2 000, diluted in blocking buffer;
- peroxidase substrate (e.g. KPL TrueBlue, SeraCare);
- Vero-E6 cells;
- Calu-3 cells;
- culture medium for Vero-E6 cells;
- culture medium for Calu-3 cells;
- medium 1% FBS;
- SARS-CoV-2 stock.

### Method

#### Preparation of cell plates

- Prepare cells ahead of time, depending on the cell line used. Prepare 96-well flat-bottom plates.
- Seed 50 000 Vero-E6 cells (medium) or 40 000 Calu-3 cells (medium) per well in a 96-well plate

#### Titration

- When the plates are 90% confluent, prepare virus dilutions in a deep-well plate.
  - A – 1:5 with 2 250 µL medium 1% FBS + 250 µL SARS-CoV-2
  - B – 1:10 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:5 dilution
  - C – 1:20 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:10 dilution
  - D – 1:40 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:20 dilution
  - E – 1:80 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:40 dilution
  - F – 1:160 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:80 dilution
  - G – 1:320 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:160 dilution
  - H – 1:640 with 1 500 µL medium 1% FBS + 1 500 µL of the 1:320 dilution
    - Columns 11 and 12 serve as negative control by adding 120 µL medium 1% FBS.
- Incubate at 37 °C for one hour.
- Pipette 100 µL from each well into the cell plate.
- Incubate for six hours at 37 °C.

Comment: The incubation time may vary depending on the virus variant and laboratory protocol.

- Remove the medium.
- Add 200 µL of 10% formalin.
- Incubate for 20 minutes.
- Remove the formalin and immerse the plate in ethanol.
- Stain the infected cells.
- Remove the ethanol and wash 1x with PBS.
- Add 0.5% Triton X-100-PBS 100 µL/well.
- Incubate for 10 minutes at 37 °C.

- Block for 10 minutes at 37 °C, with 100 µL/well blocking buffer.
- Remove the blocking buffer.
- Add 50 µL/well of the primary antibody.
- Incubate for one hour at 37 °C.
- Wash 2x with PBS.
- Add 50 µL/well of the secondary antibody.
- Incubate for one hour at 37 °C.
- Wash 2x with PBS.
- Add 50 µL/well TrueBlue.
- Incubate for 5–30 minutes in the dark at RT.
- Check every five minutes whether the plaques are clearly blue.
- Wash twice with water.
- Let the plates dry.

### **Counting plaques**

- Scan and count the plaques using the CTL ImmunoSpot analyser.
- Settings: sensitivity: 230; background balance: 40; diffuse processing: large; 'fiber' removal and ROI-touching removal: checked; minimum spot size: 0.0002 mm<sup>2</sup>; maximum spot size: 9.6296 mm<sup>2</sup>.

These parameters are for orientation only and may need to be slightly modified. The plaque size can vary between variants.

## **2.4 RT-PCR**

### **2.4.1 SARS-CoV-2 RT-PCR**

#### **Introduction**

For RNA extraction from infected cell cultures or cell-free virus suspensions, the virus suspension needs to be mixed with a lysis buffer from a viral RNA extraction kit in a ratio defined by the manufacturer. Such buffers are for example: the RAV1 lysis buffer from MACHEREY-NAGEL, MagNA pure external lysis buffer from Roche, and the buffer AVL from QIAGEN. To completely inactivate viruses of the genus Betacoronavirus, the mixture must be incubated at 70 °C for 10 minutes.

For further information about the assay, see: references [1] and [9].

#### **Materials and reagents**

- pipettes;
- filter tips;
- total nucleic acid isolation kit;
- patient material (fluid from PCR-positive swab);
- primer;
- probes;
- fast virus master mix with UDG, custom made, (Cat:#4444434, Thermo Fisher Scientific).

#### **Method**

##### **RNA extraction**

For RNA extraction the Roche MagNA pure external lysis buffer is used, therefore 400 µL patient material (e.g. fluid from a swab from the anterior nose or from the nasopharynx) is mixed with 600 µL of MagNA pure external lysis buffer in a biological safety cabinet. The resulting 1 mL sample is used in isolation. The buffer is included in the total nucleic acid isolation kit and conditions the use of the external lysis protocol for RNA isolation.

##### **RT-PCR**

RT-PCR is done on a Roche LightCycler 480 Instrument-II with the following thermocycling protocol:

Five minutes at 50 °C, 20 seconds at 95 °C, 45 cycles (three seconds at 95 °C, 30 seconds at 60 °C).

**PCR mix**

Reagent	Volume
FVMM* (4X)	5.0 µL
Primer-probe mix	0.4 µL
PCR-grade Water	6.6 µL
RNA	8.0 µL
<b>Total</b>	<b>20.0 µL</b>

\*FVMM: Fast virus Master mix with UDG, custom-made, Thermo Fisher Scientific.

**Primer-probe (PP-)mixes****Table 4. Primers and probes for SARS-CoV-2**

Target gene	Sequence 5'→ 3'	Concentration	Fluorophore / Quencher	Final concentration in PCR
E-gene				
Fwd	ACAGGTACGTTAATAGTTAATAGCGT	20 pmol/µL		400 nM
Rev	ATATTGCAGCAGTACGCACACA	20 pmol/µL		400 nM
Probe	ACACTAGCCATCCTTACTGCGCTTCG	5 pmol/µL	FAM/BHQ1	100 nM
RdRp				
Fwd	GTGARATGGTCATGTGTGGCGG	30 pmol/µL		600 nM
Rev	CARATGTTAAASACTATTAGCATA	40 pmol/µL		800 nM
Probe1	CCAGGTGGWACRTCATCMGGTGATGC	5 pmol/µL	FAM/BHQ1	100 nM
Probe2	CAGGTGGAACCTCATCAGGAGATGC	5 pmol/µL	FAM/BHQ1	100 nM

## 2.5 Sequencing

### 2.5.1 Amplicon sequencing on the MinIon

Use new caps on the strips after every incubation/PCR step.

**cDNA synthesis**

The first step of the PCR is done with the following thermocycling protocol:

Five minutes at 65 °C, cooling at 4 °C

Reagent	1x
Random hexamer [50 µM] (Thermo Fisher Scientific N8080127)	1 µL
dNTPs [10 mM]	1 µL
RNase inhibitor [40 U/µL]	0.5 µL
RNA template	10 µL
<b>Total</b>	<b>12.5 µL</b>

Thereafter the following reagents are added, followed by the main thermocycling programme: Five minutes at 25 °C, 15 minutes at 48 °C, five minutes at 80 °C, cooling at 4 °C

Reagent	1x
5x ProtoScript II buffer (NEB Biolabs M0368S)	4 µL
DTT [0.1 M]	2 µL
ProtoScript II Reverse Transcriptase [200 U/µL]	1 µL
RNase Inhibitor [40 U/µL]	0.5 µL
<b>Total</b>	<b>20 µL</b>

**PCR amplification**

The PCR multiplex reaction is performed with the following thermocycling protocol: 30 seconds at 98 °C, 40x [15 seconds at 98 °C, 5 minutes at 63 °C], cooling

Reagent	1x
5x Q5 reaction buffer	5 µL
dNTPs [10 mM]	0.5 µL
Q5 DNA polymerase	0.25 µL
Primer pool 1 or 2 [10 µM]	1.2 µL
PCR grade water	15.55 µL
<b>Total</b>	<b>22.5 µL</b>
cDNA to be added	2.5 µL

## Sample purification

### DAY 2

Pool the multi-PCR products 1 and 2 (50 µL in total).

- Allow the AMPure XP Beads, to warm up to RT for 30 minutes.
- Vortex the beads for 10 seconds.
- Add 40 µL beads to the 50 µL of sample.
- Resuspend with pipette.
- Incubate the samples at RT for 5 min. to allow the sample to bind to the beads.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant.
- Keep the sample on the magnet and add 200 µL of 80% ethanol.
- Incubate at RT for ≥30 seconds.
- Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Keep the sample on the magnet and add 200 µL of 80% ethanol.
- Incubate at RT for ≥30 seconds.
- Spin down the sample and pipette off any residual supernatant.
- Allow to dry at RT for ~30 seconds or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 26 µL elution buffer (10 mM Tris-HCL, pH 8.0) or PCR-grade water.
- Incubate at RT for 2 minutes.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove 25 µL of the supernatant and transfer it to a new tube.

Measure the concentration on the Qubit and dilute to 65 ng in 11.5 (µL) (if the concentration is too low, use all 11.5 mL)

- Make a qubit solution: per sample 199 µL buffer+ 1 µL qubit.
- (for 220 reactions: 43.8 mL buffer and 220 µL qubit).
- Make (in a strip) 8x standard S1 (190 µL qubitsolution + 10 µL S1).
- Make (in a strip) 8x standard S2 (190 µL qubitsolution + 10 µL S2).
- For every sample: 199 µL qubitsolution + 1 µL sample.
- Vortex samples.
- Measure samples.

### Library preparation (SQK-NBD114 nanopore protocol)

Defrost the barcodes and EDTA

#### DNA Control Sample

Dilute your DNA Control Sample (DCS) by adding 105 µL Elution Buffer (EB) directly to one DCS tube. Mix gently by pipetting and spin down.

One tube of diluted DNA Control Sample (DCS) is enough for 140 samples. Excess can be stored at -20 °C in the freezer.

Take 2 DNA Control Samples along in each run. For the other samples 1 µL H<sub>2</sub>O can be added instead of DCS.

DNA repair and end-prep

Reagent	Volume
65 ng DNA (200 fmol 500 bp amplicons)	11.5 µL
Ultra II End-prep reaction buffer (green lid)	1.75 µL
Ultra II End-prep enzyme mix (green lid)	0.75 µL
Diluted DNA Control Sample (DCS) or H <sub>2</sub> O	1 µL
<b>Total</b>	<b>15 µL</b>

PCR machine: 5 minutes 20 °C, 5 minutes 65 °C

- When pause here, clean up the sample (ratio 1:1), otherwise continue with end-prep material:

Native barcode ligation

Reagent	Volume
End-prepped DNA	0.75 µL
Native Barcode (96 wells plate)	1.25 µL
Blunt/TA Ligase Master Mix (white lid)	5 µL
Nuclease-free water	3 µL
<b>Total</b>	<b>10 µL</b>

Mix by pipetting and incubate 20 minutes RT.

- Add 1 mL of EDTA (see through cap) and mix thoroughly and spin down (EDTA stops the reaction). Check color of EDTA tube (blue cap has different volumes)
- Pool the barcoded samples in a 1.5 mL Eppendorf tube:

	Volume per sample	For 24 samples	For 48 samples	For 96 samples
Total volume	11 µL	264 µL	528 µL	1,056 µL

- Add resuspended AMPure XP beads 0.8X, mix by pipetting:

	Volume per sample	For 24 samples	For 48 samples	For 96 samples
Volume of AXP	8.8 µL	211 µL	422 µL	845 µL

- Incubate the samples at RT for 10 minutes on a Hula mixer to allow the sample to bind to the beads.
- Spin down the sample.
- Place the samples in a magnetic particle collector to capture the beads (5 minutes). Incubate until the liquid is clear.
- Carefully remove and discard the supernatant
- Keep the sample on the magnet and add 700 µL of 80% ethanol.
- Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Keep the sample on the magnet and add 700 µL of 80% ethanol.
- Spin down the sample and pipette off any residual supernatant.
- Allow to dry at RT for ~30 seconds or until the beads are dry.
- Remove the samples from the magnet
- Resuspend the DNA using 32 µL PCR-grade water.
- Incubate at 37 °C for 10 minutes.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove 31 µL of the supernatant and transfer it to a new DNA LoBind tube (possible to store samples o/n at 4 °C).

Adapter ligation and clean-up

Thaw one tube of S Fragment Buffer (SFB) (= Small Fragment Buffer, see-through lid)

Reagent	Volume
Pooled barcoded sample DNA	30 µL
Native adapter (NA)	5 µL
NEBNext Quick Ligation Reaction Buffer (5x) (red lid)	10 µL
Quick T4 DNA Ligase (red lid)	5 µL
<b>Total</b>	<b>50 µL</b>

Mix by flicking the tube and incubate 20 minutes RT.

- Add 40 mL of resuspended AMPure XP beads, mix by pipetting.

- Incubate the samples at RT for 10 minutes on a Hula mixer to allow the sample to bind to the beads.
- Spin down the sample.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant.
- Take the sample of the magnet and wash the beads with 125  $\mu\text{L}$  S Fragment Buffer (SFB) by flicking the tube.
- Put the sample on the magnet and carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Repeat the wash step.
- Spin down the tube, place back on the magnet and pipette off any residual supernatant.
- Allow to dry at RT for  $\sim 30$  seconds or until the beads are dry.
- Remove the samples from the magnet
- Resuspend the DNA using 15  $\mu\text{L}$  elution buffer Elution buffer from Nanopore (or PCR grade water).
- Incubate at 37  $^{\circ}\text{C}$  for 10 minutes, every two minutes, gently flick the tube.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear (at least 1 minute).
- Carefully remove 15  $\mu\text{L}$  of the supernatant and transfer it to a new DNA LoBind tube.

Measure the concentration on the Qubit.

- Make a library to 12  $\mu\text{L}$  at 10-20 fmol

### MinIon preparation

(check the website to keep the protocol updated)

- Store the reagents on ice

### Flow cell priming mix

Reagent	One reaction ( $\mu\text{L}$ )
Bovine Serum Albumin (BSA 50 mg/mL)	5
FCT (Flow Cell Tether)	30
FCF (Flow Cell Flush) (=1 tube)	1 170
<b>Total</b>	<b>1 205</b>

### Preparation of library for loading:

Reagent	One reaction ( $\mu\text{L}$ )
Sequencing Buffer (SB red lid)	37.5
Library beads (LB (mix before adding) (red lid)	25.5
DNA library (50 ng) (10-20 fmol à 5 ng)	12
<b>Total</b>	<b>75</b>

*Preparation to load the flow cell:*

- Place the flow cell on the grid ion
- Do a flowcell check (minimal amount of pores = 800)
- Flip back the MinIon lid and slide the priming port cover clockwise so that the priming port is visible
- Draw back a small volume to remove any bubbles
  - 1) Set a P1000 pipette to 200  $\mu\text{L}$
  - 2) Insert the tip into the priming port
  - 3) Turn the wheel until the dial shows 220-230  $\mu\text{L}$  (to get some volume out of flow cell)
- Load 800  $\mu\text{L}$  of the priming mix into the flow cell, avoid air bubbles
- Wait 5 minutes
- Gently lift the SpotOn sample port cover to make the SpotOn sample port accessible
- Load 200  $\mu\text{L}$  of the priming mix into the flow cell via the priming port (P1000 pipette)
- Mix the prepared library gently by pipetting up and down.
- Add 75  $\mu\text{L}$  of sample to the flow cell via the SpotOn port (drop wise) (P200 pipette).
- Close the ports
- Start the run:
  - Name run: Viro\_Run\_nr (check database for nr)
  - Select 16h run time (run options)
  - Select the kit used (LSK-114, SQK-NBD114.96)
  - Run length: 16h
  - Minimal reads length: 200 bp

Basecalling: on (super accurate), trim barcodes, both ends, mid read barcode filtering)  
 Alignment: off  
 FAST5: off  
 FASTQ: on (output every hour)

## 2.5.2 Amplicon sequencing on the Oxford Nanopore platform, starting from RNA

### Materials and reagents

- pipettes;
- filter tips;
- reaction vessels;
- dNTPs;
- RNase inhibitor;
- RNA template;
- enzyme;
- reaction buffer;
- primers;
- RFA.

### Method

Use new caps on the strips after every incubation/PCR step.

### cDNA synthesis

The first step of the PCR is done with the following thermocycling protocol:

Five minutes at 65 °C, cooling at 4 °C

Reagent	1x
Random hexamer [50 µM] (Thermo Fisher Scientific N8080127)	1 µL
dNTPs [10 mM]	1 µL
RNase inhibitor [40U/µL]	0.5 µL
RNA template	10 µL
<b>Total</b>	<b>12.5 µL</b>

Thereafter the following reagents are added, followed by the main thermocycling program:

Five minutes at 25 °C, 15 minutes at 48 °C, five minutes at 80 °C, cooling at 4 °C

Reagent	1x
5x ProtoScript II buffer (NEB Biolabs M0368S)	4 µL
DTT [0.1 M]	2 µL
ProtoScript II Reverse Transcriptase [200 U/µL]	1 µL
RNase Inhibitor [40 U/µL]	0.5 µL
<b>Total</b>	<b>20 µL</b>

### PCR amplification

For the PCR amplification, the [primers of ARTIC version 4.1](https://github.com/artic-network/primer-schemes/tree/master/nCoV-2019/V4.1) are used. <https://github.com/artic-network/primer-schemes/tree/master/nCoV-2019/V4.1>

The PCR multiplex reaction is performed with the following thermocycling protocol: 30 seconds at 98 °C, 40x [15 seconds at 98 °C, 5 minutes at 65 °C], cooling.

Reagent	1x
5x Q5 reaction buffer	5 µL
dNTPs [10 mM]	0.5 µL
Q5 DNA polymerase	0.25 µL
Primer pool 1 or 2 [10 µM]	1.2 µL
PCR grade water	15.55 µL
<b>Total</b>	<b>22.5 µL</b>
cDNA to be added	2.5 µL

Pool the multiplex PCR products 1 and 2 (50 µL in total).

### Sample purification

- Allow the AMPure XP beads, to warm up to RT for 30 minutes.
- Vortex the beads for 10 seconds.
- Add 40  $\mu\text{L}$  beads to 50  $\mu\text{L}$  sample (add 10  $\mu\text{L}$  water).
- Mix thoroughly by vortexing for 10 seconds.
- Incubate the samples at RT for five minutes to allow the samples to bind to the beads.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant.
- Keep the samples on the magnet and add 200  $\mu\text{L}$  of 80% ethanol.
- Incubate at RT for  $\geq 30$  seconds.
- Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Keep the samples on the magnet and add 200  $\mu\text{L}$  of 80% ethanol.
- Incubate at RT for  $\geq 30$  seconds.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples to dry at RT for  $\sim 30$  seconds, or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 53  $\mu\text{L}$  elution buffer (10 mM Tris-HCl, pH 8.0) or PCR-grade water.
- Remove the samples from the magnet.
- Vortex the beads for 10 seconds.
- Incubate at RT for two minutes.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear. Carefully remove 53  $\mu\text{L}$  of the supernatant and transfer it to a new tube.

Measure the concentration on the qubit and dilute it to 100 ng in 53.5  $\mu\text{L}$  (if the concentration is too low, use 52  $\mu\text{L}$  and add 1.5  $\mu\text{L}$  water).

### Library preparation (SQL-LSK109, Oxford Nanopore protocol)

#### DNA repair and end-prep

Reagent	Volume
DNA	53.5 $\mu\text{L}$
Ultra II End Prep reaction buffer	3.5 $\mu\text{L}$
Ultra II End Prep enzyme mix	3.0 $\mu\text{L}$
<b>Total</b>	<b>60 <math>\mu\text{L}</math></b>

PCR machine: Five minutes at 20  $^{\circ}\text{C}$ , and five minutes at 65  $^{\circ}\text{C}$ .

- Add 60  $\mu\text{L}$  of resuspended AMPure XP beads. Mix by pipetting.
- Incubate the samples at RT for five minutes on a HulaMixer to allow the samples to bind to the beads.
- Spin down the samples.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant.
- Keep the samples on the magnet and add 200  $\mu\text{L}$  of 80% ethanol.
- Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Keep the samples on the magnet and add 200  $\mu\text{L}$  of 80% ethanol.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples to dry at RT for  $\sim 30$  seconds, or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 25  $\mu\text{L}$  PCR-grade water.
- Incubate at RT for two minutes.
- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear. Carefully remove 22.5  $\mu\text{L}$  of the supernatant and transfer it to a new DNA LoBind tube.

Measure the concentration on the qubit and dilute it to 500 ng in 22.5  $\mu\text{L}$ .

#### Native barcode ligation

Reagent	Volume
500 ng end-prepped DNA	22.5 $\mu\text{L}$
Native barcode	2.5 $\mu\text{L}$
Blunt/TA Ligase Master Mix	25 $\mu\text{L}$
<b>Total</b>	<b>50 <math>\mu\text{L}</math></b>

Mix by pipetting and incubating for 10 minutes at RT.

- Add 50  $\mu\text{L}$  of resuspended AMPure XP beads. Mix by pipetting.
- Incubate the samples at RT for five minutes on a HulaMixer to allow the samples to bind to the beads.
- Spin down the samples.

- Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
  - Carefully remove and discard the supernatant.
  - Keep the samples on the magnet and add 200  $\mu\text{L}$  of 80% ethanol.
  - Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
  - Keep the samples on the magnet and add 200  $\mu\text{L}$  of 80% ethanol.
  - Spin down the samples and pipette off any residual supernatant.
  - Allow the samples to dry at RT for  $\sim 30$  seconds, or until the beads are dry.
  - Remove the samples from the magnet.
  - Resuspend the DNA using 26  $\mu\text{L}$  PCR-grade water.
  - Incubate at RT for two minutes.
  - Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
- Carefully remove 26  $\mu\text{L}$  of the supernatant and transfer it to a new DNA LoBind tube.

Measure the concentration on the qubit and dilute it to 350 ng in 65  $\mu\text{L}$  (for 48 samples: 7.3 ng/sample, for 96 samples: 3.65 ng/sample). If the pooled sample volume exceeds 65  $\mu\text{L}$ , add the same volume of AMPure XP beads, elution buffer in 65  $\mu\text{L}$ .

### Adapter ligation and clean-up

Thaw one tube of Short Fragment Buffer (SFB).

Reagent	Volume
700 ng pooled barcoded sample DNA	65 $\mu\text{L}$
Adapter Mix II Expansion (AMII)	5 $\mu\text{L}$
NEBNext Quick Ligation Reaction Buffer (5x)	20 $\mu\text{L}$
Quick T4 DNA Ligase	10 $\mu\text{L}$
<b>Total</b>	<b>100 <math>\mu\text{L}</math></b>

Mix by flicking the tube and incubating for 10 minutes at RT.

- Add 80  $\mu\text{L}$  of resuspended AMPure XP beads. Mix by pipetting.
- Incubate the samples at RT for five minutes on a HulaMixer to allow the samples to bind to the beads.
- Spin down the samples.
- Place the samples in a magnetic particle collector to capture the beads.
- Incubate until the liquid is clear.
- Carefully remove and discard the supernatant.
- Keep the samples on the magnet and wash the beads with 250  $\mu\text{L}$  SFB.
- Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Keep the samples on the magnet and wash the beads with 250  $\mu\text{L}$  SFB.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples to dry at RT for  $\sim 30$  seconds, or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 15  $\mu\text{L}$  elution buffer.
- Incubate at RT for 10 minutes.
- Place the samples in a magnetic particle collector to capture the beads.
- Incubate until the liquid is clear.

Carefully remove  $\sim 15$   $\mu\text{L}$  of the supernatant and transfer it to a new DNA LoBind tube.

### Preparation to load the flow cell<sup>i</sup>

Store the reagents on ice.

Flow cell priming was prepared:

Reagent	1x
FLT	30 $\mu\text{L}$
1 tube Flush Buffer (FB)	volume not known

<sup>i</sup> Flow cell priming mix was prepared.

Check the [Oxford Nanopore Technologies website](https://www.nanoporetech.com/support/) to keep the protocol updated.

The library was prepared for loading:

Reagent	1x
Sequencing Buffer (SQB)	45.5 µL
Loading Beads (LB) (mix before adding)	25.5 µL
DNA library (50 ng)	~4 µL
<b>Total</b>	<b>75 µL</b>

The library was loaded on to the flow cell:

- Place the flow cell on the GridION.
- Flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.
- Draw back a small volume to remove any bubbles.
  - Set a P1000 pipette to 200 µL.
  - Insert the tip into the priming port.
  - Turn the wheel until the dial shows 220–230 µL (to get excess volume out of the flow cell).
- Load 800 µL of the priming mix into the flow cell. Avoid air bubbles.
- Wait for five minutes.
- Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
- Load 200 µL of the priming mix into the flow cell via the priming port.
- Mix the prepared library gently by pipetting up and down.
- Add 75 µL of the sample to the flow cell via the SpotON port (drop wise).
- Close the ports.
- Start the run:
  - Name the run.
  - Select a run time of 16 hours (run options).
  - Select the kit used (SQK-LSK109/LSK110).
  - Run length: 16 hours.
  - Minimal reads length: 200 bp.
  - Basecalling: on (high accuracy).
  - Alignment: off.
  - FAST5: off.
  - FASTQ: on (GZip, 32 000 reads per file).

## 2.6 Plaque reduction/focus-forming assays

### 2.6.1 Testing human serum specimens for neutralising antibodies against SARS-CoV-2, by means of PRNT (using crystal violet)

#### *Introduction*

This protocol describes the method to test human serum specimens for neutralising antibodies against SARS-CoV-2.

Be aware of the following safety instructions, since specimens and controls are potentially infectious:

- Use a Class II biosafety cabinet in a BSL-3 laboratory.
- Clean up spilled reagents immediately, and decontaminate the surface if necessary.
- In case of obvious contamination, initiate decontamination immediately.
- Use pipette tips with filters to handle samples and controls.

For further information about the assay, see: references [21], [22], and [23].

#### *Materials and reagents*

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- 24-well plates;
- 1.5 mL reaction tubes with safe-lock lid;
- OptiPRO Serum Free Medium (Thermo Fisher Scientific);
- 2x DMEM (Thermo Fisher Scientific);
- DMEM with 10% FBS;
- Avicel RC-581 (2.4%) stock solution (FMC Biopolymer International);
- 6% formaldehyde in PBS;
- crystal violet solution (0.2%);

- Vero-E6 cells, tested negative for mycoplasma and PIV5;
- SARS-CoV-2 virus stock.

## Method

### Principle

- Obtain the serum.
- Heat inactivate the sera at 56 °C for 30 minutes in a water bath.
- Incubate the serum dilution series with SARS-CoV-2 (50 PFU/100 µL) for one hour at 37 °C and 5% CO<sub>2</sub>.
- Incubate serum/virus samples on seeded Vero-E6 cells for one hour at 37 °C and 5% CO<sub>2</sub>.
- Overlay cells with 1.2% Avicel/DMEM for three days at 37 °C and 5% CO<sub>2</sub>.
- Fix the cells with 6% formaldehyde.
- Stain the cells with crystal violet and evaluation of PFU.

### Lower limit of quantification (LLOQ)

Measuring points: Evaluate after three days by formaldehyde fixation followed by staining of the cell monolayer with crystal violet.

SARS-CoV-2: Titre of 1:20 with reduction of 50% or 90% of maximum virus plaques generated.

### Pre-analytics

Test material: Human serum/plasma.

Transport and storage of primary samples/archiving: ≤24 hours at 20 °C, >24 hours to maximum, one year at -20° C in the freezer.

Storage of processed samples: ≤1 week at -20 °C.

### Calibration

Adjust the PFU by virus dilution series after preparation of new virus stocks (reference range maximum: 50 PFU/24-well).

Use a reference serum with defined endpoint titre to check the reagents.

Comment: The assay can be calibrated with the 1st and 2nd WHO IS for anti-SARS-CoV-2 antibodies (NIBSC 21/340 and 21/338).

### Quality control procedures

- Negative control (NC): 'DMEM only'
- Virus control (VC): 'Virus only'
- Positive control (PC): Neutralising SARS-CoV-2 serum

### Performance of the analysis

- Heat inactivate the sera: 56 °C for 30 minutes in a water bath.
- Incubate a serum dilution series with SARS-CoV-2.
- Prepare a serum dilution series: 1:10 – 1:640.
- Prepare a virus dilution: 50 PFU/100 µL.
- Incubate the serum dilution series with SARS-CoV-2 (110 µL + 110 µL) for one hour at 37 °C (double preparation).
- Incubate serum+virus samples on seeded Vero-E6 cells: seeding of cells one day before assay (1.5x10<sup>5</sup> cells/24-well).
- Incubate serum+virus samples (200 µL) on the cells for one hour at 37 °C and 5% CO<sub>2</sub>.
- Overlay cells with Avicel solution in DMEM: 1.2% Avicel in DMEM (500 µL/24-well).
- Incubate for three days at 37 °C and 5% CO<sub>2</sub>.
- Fix the cells: 6% formaldehyde in PBS for at least 30 minutes.
- Stain the cell lawn with crystal violet solution: evaluation of the PFU.

### Influencing factors/interfering factors

- Inadequate storage and handling of specimen materials and reagents.
- Non-compliance with volumes and reaction times.
- Contamination of cell cultures.

### Determination of results/conversion factors/units

Calculation of serum dilutions with 50% or 90% reduction of PFUs compared to positive control ('virus only').

## Result evaluation/result interpretation

Technical validation: The evaluation of the results for the test parameters is performed by the responsible technical staff.

## 2.6.2 Antibody-based plaque reduction/focus-forming assays using the Cellular Technology Limited (CTL) ImmunoSpot analyser

### Introduction

This procedure describes the method to quantify the neutralising antibodies against SARS-CoV-2 in serum, using a focus reduction neutralisation test (FRNT). In an FRNT, a constant amount of SARS-CoV-2 is mixed with a serial dilution of a (patient) material (e.g. serum). After an incubation time, the mixture is added to cultured cells. The number of infected cells (foci) is a measure of the amount of infectious virus in the mix. The presence of SARS-CoV-2 neutralising antibodies prevents infection of the cells and reduces the number of foci. The FRNT<sub>50</sub> (focus reduction neutralisation titre) is defined as the dilution at which 50% of the virus is neutralised.

### Materials and reagents

- PIPETBOY
- serological pipettes;
- pipettes;
- filter tips;
- falcon tubes;
- 96-well flat-bottom plate;
- 96-well U-bottom plate;
- PBS;
- FBS
- trypsin;
- 10% formalin;
- 70% ethanol;
- 0.5% Triton X-100-PBS;
- blocking buffer;
- 20% (w/v) skimmed milk in PBS;
- first antibody: SARS-CoV-2 rabbit polyclonal antibody (Catalog # 40143-T62, Sino Biological) 1:1 000 diluted in blocking buffer;
- second antibody: polyclonal goat anti-rabbit immunoglobulins/HRP (Catalog # P044801-2, Agilent), 1:2 000 diluted in blocking buffer;
- KPL TrueBlue Peroxidase Substrate (Catalog # 5510-0030);
- Vero-E6 cells;
- Calu-3 cells;
- growth medium (10% FBS) for Vero-E6 cells and Calu-3 cells;
- Maintenance medium (1% FBS) for Vero-E6 cells and Calu-3 cells;
- SARS-CoV-2 virus stock;
- Neutralising serum.

### Method

#### Day -1 (for Vero-E6 cells) or day 6 (for Calu-3 cells)

- The cells have to be trypsinised. Therefore the procedure is as follows:
  - Pre-warm medium, trypsin and PBS in the water bath at 37 °C.
  - Remove the medium from the cells in the T175 flask.
  - Wash the cells with PBS to remove FBS.
  - Add 10 mL of trypsin and leave for 30 to 60 seconds.
  - Check under the microscope to see if cells are 'rounding'.
  - Remove most of the trypsin and place the flask in the incubator at 37 °C, for 15 to 20 minutes.
  - Gently tap the bottle to detach the cells.
  - Collect the cells in 10 mL of growth medium to stop the trypsin.
  - Centrifuge at 200xg for five minutes.
  - Discard the supernatant and resuspend the pellet in 10 mL of growth medium.
- Count the cells and adjust the volume to 5.0x10<sup>5</sup> cells/mL (Vero-E6) or 4.0x10<sup>5</sup> (Calu-3).
- Seed the cells in a 96-well flat-bottom plate in 100 µL/well (50 000/40 000 cells per well).

#### Day 0

- Heat inactivate 50 µL serum for 30 minutes at 56 °C.

- Each plate includes positive (neutralising serum) and a back-titration of the virus stock (used to calculate the PRNT<sub>50</sub>).
- Dispense the maintenance medium (1% FBS) for the test plates according to Table 5.

**Table 5. Serial dilution scheme for plaque reduction assay**

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		+serum	Virus ctrl
	1	2	3	4	5	6	7	8	9	10	11	12
A	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	108 (+12)	120
B	60	60	60	60	60	60	60	60	60	60	60	60
C	60	60	60	60	60	60	60	60	60	60	60	60
D	60	60	60	60	60	60	60	60	60	60	60	60
E	60	60	60	60	60	60	60	60	60	60	60	60
F	60	60	60	60	60	60	60	60	60	60	60	60
G	60	60	60	60	60	60	60	60	60	60	60	60
H	60	60	60	60	60	60	60	60	60	60	60	60

Volume in  $\mu\text{L}$ ; ctrl : control; black: volume for maintenance medium; blue: volume for serum

Prepare the serial serum dilution in a U-bottom plate:

- Add 12  $\mu\text{L}$  of serum to designated wells (blue in the schedule above).
- Transfer 60  $\mu\text{L}$  from row A to row B and resuspend at least eight times.
- Change the pipette tips.
- Transfer 60  $\mu\text{L}$  from row B to row C and resuspend at least eight times.
- Change the pipette tips.
- Repeat until row H and discard 60  $\mu\text{L}$  from row H.
- The final volume should be 60  $\mu\text{L}$  uniformly.

Add the virus (in a Class II biosafety cabinet in a BSL-3 laboratory):

- Thaw the SARS-CoV-2 stock and dilute it in the maintenance medium (1% FBS) to 150–400 PFU.
- Add 60  $\mu\text{L}$  SARS-CoV-2 diluted virus to all wells with serum dilution. The first serum dilution is now 1:20. All the wells have a final volume of 120  $\mu\text{L}$ .
- Incubate the serum/virus mix for one hour at 37 °C.

Add the virus/serum mix to the cells:

- Remove the medium from the cells.
- Transfer 100  $\mu\text{L}$  of the serum/virus mix to the cells.
- Incubate for six hours at 37 °C.
- Remove the mix from the cells.
- Fix the cells with 200  $\mu\text{L}$  10% formalin for 20 minutes.
- Remove the formalin and immerse in 70% ethanol.

Staining the plaques:

- Remove the ethanol and wash 1x with PBS.
- Add 100  $\mu\text{L}$ /well 0.5% Triton X-100-PBS.
- Incubate for 10 minutes at 37 °C.
- Block for 10 minutes at 37 °C with 100  $\mu\text{L}$ /well blocking buffer.
- Remove the blocking buffer.
- Add 50  $\mu\text{L}$ /well of the primary antibody.
- Incubate for one hour at 37 °C.
- Wash 2x with PBS.
- Add 50  $\mu\text{L}$ /well of the secondary antibody.
- Incubate for one hour at 37 °C.
- Wash 2x with PBS.
- Add 50  $\mu\text{L}$ /well peroxidase substrate (e.g. TrueBlue).
- Incubate for 5–30 minutes in the dark at RT.
- Check every five minutes whether the plaques are clearly blue.
- Wash 2x with water.
- Let the plates dry.

Counting the plaques:

- Scan and count the plaques using the CTL ImmunoSpot analyser.
- Settings: sensitivity: 230; background balance: 40; diffuse processing: Large; fiber removal and ROI-touching removal: checked; minimum spot size: 0.0002 mm<sup>2</sup>; maximum spot size: 9.6296 mm<sup>2</sup>.

Data processing:

- The positive control serum (in column 11 on each plate) is included to check whether the titre corresponds to the known value. A silver standard can be determined to calculate the IU.
- Virus control (negative control) (in column 12) is included on each plate. The mean of this is used to determine the PRNT<sub>50</sub> titre.
- Every two rows (1–2, 3–4, 5–6, 7–8 and 9–10) is a patient serum. The mean of these two rows is included in the titre determination: if the titre is 20 or higher, it is considered positive.

## 2.6.3 Virus Neutralisation Test SARS-CoV-2

### Introduction

A virus neutralisation assay can be performed to detect neutralising antibodies to SARS-CoV-2 in sera. In this variant of the plaque assay, virus entry into the cell is prevented by binding neutralising antibodies to the surface of the virus. As a result, viral entry is reduced and the number of plaques in the cell culture is decreased.

### Materials and reagents

- pipettes;
- filter tips;
- 96-well flat-bottom plates;
- 96-well U-bottom plates;
- Vero-E6 medium;
- sera to be tested;
- titrated SARS-CoV-2 virus;
- control serum that contains anti-SARS-CoV-2 neutralising antibodies;
- Vero-E6 cells (required concentration = 1.8 - 2x10<sup>5</sup> cells/mL).

### Method

#### Day-1 or 0: Sera inactivation

Inactivate 25 µL serum for 30 minutes at 56 °C, including the control serum that contains anti-SARS-CoV-2 neutralising antibodies.

#### Day 0: Virus Neutralisation Test (VNT)

- Pre-warm the Vero-E6 medium.
- Dilute the inactivated sera 1:5 in the Vero-E6 medium by adding 100 µL medium to 25 µL inactivated serum from step 1.
- Add the medium to a plate according to Table 6.
  - Add 25 µL Vero-E6 medium to all the wells.
  - Add an extra 25 µL to the wells of columns 11 and 12 (cell control (CC)).
- Add 25 µL diluted serum (each serum in duplicate) to wells A1–A10 (serum control (SC)) and resuspend.
- Add 25 µL diluted serum (each serum in duplicate) to B1–B10 and resuspend.
- Make a 1:2 serum dilution by taking 25 µL of the wells B1–B10, and adding that to the wells C1–C10.
  - Resuspend and repeat up to and including H1–H10 (change the pipette tips between each row).
  - Discard 25 µL after row H, so the end volume in H1–H10 is 25 µL.
- Remove 25 µL of the wells A1–A10 and add 25 µL fresh Vero-E6 medium to A1–A10 (SC).
- Dilute the virus in the Vero-E6 medium (approximately 2 mL/plate); e.g. aim for 25 µL containing 100 TCID<sub>50</sub> virus.
- Add 25 µL virus dilution to all the wells, except wells A1–A10 (SC) and columns 11 and 12 (CC).
- Incubate all the plates for one hour at 35 °C and 5% CO<sub>2</sub>.

Comment: In some protocols, the incubation temperature is 37 °C.

- Add 100 µL cell suspension per well for the whole plate.
- Per well 1.8x10<sup>4</sup> cells are needed
  - 1.8x10<sup>5</sup> cells/mL
  - 1.8x10<sup>6</sup> cells/plate (= 10 mL)
- Incubate for three days at 35 °C and 5% CO<sub>2</sub>.

Comment: In some protocols, the incubation temperature is 37 °C.

**Table 6. Serial dilution scheme for virus neutralisation test**

Dilution		Serum A	Serum A	Serum B	Serum B	Serum C	Serum C	Serum D	Serum D	Serum E	Serum E	CC**	CC**
		1	2	3	4	5	6	7	8	9	10	11	12
1:10 SC*	A	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:10	B	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:20	C	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:40	D	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:80	E	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:160	F	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:320	G	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL
1:640	H	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	50 µL

\* SC: serum control \*\* CC: cell control

**Day 0: Virus titre control – back titration**

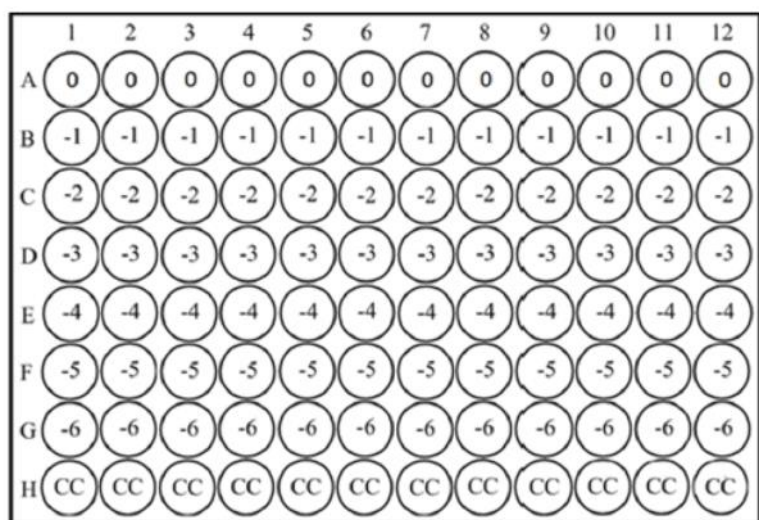
- Pre-warm the Vero-E6 medium.
- Add 25 µL Vero-E6 medium to a 96-well plate (flat-bottom) in rows A to G.
- Add 50 µL Vero-E6 medium to a 96-well plate (flat-bottom) in row H (CC).
- Take a sterile 96-well-plate (U-bottom) for dilutions and add 180 µL Vero-E6 medium to the wells of rows B to H.
- Pipette 180 µL of the virus dilution that was prepared for the VNT (100 TCID<sub>50</sub>/25 µL) in the wells of row A of the dilution plate.
- Pipette 20 µL from row A to B and resuspend (change pipette tips before the next step).
- Pipette 20 µL from row B to C and resuspend (change pipette tips before the next step).
- Repeat until row G is reached.
- Pipette 25 µL from all the wells of rows A to G to all the corresponding wells of the flat-bottom plate (see Figure 1).
- Incubate the plate for one hour at 35 °C and 5% CO<sub>2</sub>.

Comment: In some protocols, the incubation temperature is 37 °C.

- Add 100 µL cell suspension per well
  - Per well 1.8x10<sup>4</sup> cells needed
    - 1.8x10<sup>5</sup> cells/mL
    - 1.8x10<sup>6</sup> cells/plate (= 10 mL)
- Incubate for three days at 35 °C and 5% CO<sub>2</sub>.

Comment: In some protocols, the incubation temperature is 37 °C.

**Figure 1. Design of flat-bottom plate for virus neutralisation test**



**Day 3: Read-out of plates**

- Evaluate the wells under a microscope: positive for SARS-CoV-2 ('1' = CPE) or negative ('0' = no CPE).
- VNT<sub>90</sub>: a well is positive (1) if less than 90% of the cell layer is intact ( $\geq 1$  plaque(s)).
- VNT<sub>50</sub>: a well is positive (1) if less than 50% of the cell layer is intact.
- Back titration: a well is positive (1) if there is any indication for CPE; a well is negative (0) if the complete cell layer is intact.

**Day 3: Back titration**

- VNT is only considered a valid test if the back titration titre is between 50 and 200 TCID<sub>50</sub>/25  $\mu$ L.

Note: for a back titration to be exactly 100 TCID<sub>50</sub> all the wells of rows A and B should be positive (1) and six wells of row C should be positive (1).

**Day 3: Cell and serum controls**

- None of the wells of the serum or cell controls can show any signs of CPE or toxic effects on the cell layer. Otherwise, the VNT is not considered valid.

**Day 3: Control serum that contains anti SARS-CoV-2 neutralising antibodies**

- This serum is taken along in every VNT assay.
  - Based on previously determined VNT tests it should never differ from the previous results more than two dilution steps.
  - If this is the case, the VNT should be repeated.

## 2.6.4 Virus Neutralisation Test SARS-CoV-2 with staining

**Caution**

This test should only be executed under BSL3 conditions, except for the preparation steps (day -1 and day 0 up to and including the preparation of cell plates in between incubations) and the staining after fixation of the cells, these steps should be executed under BSL2 conditions. All steps that can be performed in a biosafety cabinet should be performed there.

You will be handling infectious materials. Follow all biosafety measures that apply to microbiological laboratories.

Disinfect all visible contaminations while executing the test and disinfect all potentially contaminated surfaces after using the test. Use a 1% dichloroisocyanurate dehydrate (w/v) in water and let it soak for two minutes, followed by ethanol 70% (v/v) in water.

Treat different chemicals in this assay with the highest caution.

For further information about the assay, see: references [24], [25], and [26].

**Introduction****1.1 Goal**

Determine the neutralising antibody titers against SARS-CoV-2 in sera.

**1.2 General information**

This is a virus neutralisation test executed in a 96-wells plate, using threefold serum dilutions to assess their capability to neutralise 100TCID<sub>50</sub> of infectious SARS-CoV-2. This test was initially developed using the SARS-CoV-2 wildtype strain but can also be used for other strains or isolates, as long as the titer is known.

**1.3 Relevant for**

Laboratory personnel IDS/VIR. Personnel working with the virus must be eligible for BSL-3 work and have the appropriate training.

**Materials and reagents**

- Incubator 37 °C, 5% CO<sub>2</sub>;
- Incubator 35 °C, 5% CO<sub>2</sub>;
- Water bath or heat block 56 °C;
- Cryovials;
- Sterile F-bottom 96-wells plates;
- Sterile U-bottom 96-wells plates;
- (Multichannel) pipettes;
- Pipetboy;

- Serological pipettes;
- Vortex shaker;
- Centrifuge;
- Washer BioTek E1x50 (IDS/A008) or BioTek 50TS (6184) or BioTek 405LS (plancode: 97090);
  - Thermo Scientific Multiskan FC (ELISA reader) (IDS/A706)
  - Filter 1: 450 nm
  - Filter 2: 620 nm
- Medium (2%): DMEM + 1 mM pyruvate, 2% FBS and 1% pen/strep;
- Medium (5%): DMEM + 1 mM pyruvate, 5% FBS and 1% pen/strep for growth of Vero-E6 cells;
- 10% Formaldehyde: 37% Formaldehyde and PBS;
- Permeabilization buffer: PBS + 0,02% Triton X-100.

Comment: At least 10 mL of permeabilisation buffer is needed per plate. This solution must be prepared fresh

- Dilution buffer: PBS + 1% (w/v) BSA:

Comment: At least 10 mL of permeabilisation buffer is needed per plate. This solution must be prepared fresh

- Stop solution: 0.5 M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) in sterile water:

Comment: At least 10 mL of permeabilisation buffer is needed per plate. This solution must be prepared fresh

- 70% Ethanol;
- PBS;
- Triton X-100;
- Aquadest;
- BSA;
- TMB2M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>).

### Biological material

- Human sera;
- Internal control. The internal control is prepared from a pool of different sera containing IgG antibodies against SARS-CoV-2;
- SARS-CoV-2 stock cultured for VNT and titrated for analysis by staining;
- Primary antibody: 'SARS-CoV-2 nucleocapsid mouse mAb antibody';
- Secondary antibody: "Invitrogen A16078; goat anti-mouse IgG/HRP highly cross-absorbed;
- Vero-E6 cell suspension prepared for neutralisation test.

### Method

All steps should be performed in a validated Biosafety Cabinet Type 2. Staining can be performed outside of a Biosafety Cabinet Type 2.

## Plate Layouts

**Table 7. Example plate layout 5 samples, dilution range 1:10 – 1:21 870.**

	1	2	3	4	5	6	7	8	9	10	11	12
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		VC	CC
A	10	10	10	10	10	10	10	10	10	10	VC	CC
B	30	30	30	30	30	30	30	30	30	30	VC	CC
C	90	90	90	90	90	90	90	90	90	90	VC	CC
D	270	270	270	270	270	270	270	270	270	270	VC	CC
E	810	810	810	810	810	810	810	810	810	810	VC	CC
F	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	VC	CC
G	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	VC	CC
H	21 870	21 870	21 870	21 870	21 870	21 870	21 870	21 870	21 870	21 870	VC	CC

CC = cell control, VC = Virus control

**Table 8. Example plate layout 6 samples, dilution range 1:10 – 1:7 290.**

	1	2	3	4	5	6	7	8	9	10	11	12
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
A	10	10	10	10	10	10	10	10	10	10	10	10
B	30	30	30	30	30	30	30	30	30	30	30	30
C	90	90	90	90	90	90	90	90	90	90	90	90
D	270	270	270	270	270	270	270	270	270	270	270	270
E	810	810	810	810	810	810	810	810	810	810	810	810
F	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430	2 430
G	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290	7 290
H	VC	VC	VC	VC	VC	VC	CC	CC	CC	CC	CC	CC

CC = cell control, VC = Virus control

## Day -1 or Day 0: Preparation (BSL2)

- Sera inactivation
  - Pipette 20 µL serum in a 0.5 mL cryovial and inactivate for 30 minutes at 56 °C;
  - Centrifuge inactivated sera to spin down any suspension left inside the cap;
  - In case this preparation is done on day -1: store the diluted inactivated sera in the fridge at 2-8 °C for use on the following day.
- Cell seeding
  - Prepare a cell suspension containing 1 x 10<sup>5</sup> Vero-E6 cells/mL. One 96-well plate requires approximately 10 mL of cell suspension. Prepare a few mL extra to make pipetting easier;
  - Add 100 µL of cell suspension and well. Per well 1.0<sup>4</sup> cells/well are seeded;
  - Incubate overnight at 37 °C + 5% CO<sub>2</sub>.

Comment: In case that a serum or a panel of different sera is tested multiple times (e.g. with different virus strains), it is advised to inactivate a larger volume of the serum which can be aliquoted, frozen, and stored. In that way, individual aliquots can be thawed prior to each experiment. This ensures a more homogeneous sample handling and prevents multiple freeze-thawing rounds of the sera.

## Day 0: Preparation (BSL2)

- Serial dilutions sera
  - Make a 1:5 dilution by adding 80 µL medium (+ 2% FBS + 1% Pen/Strep) to the 20 µL inactivated sera
  - Pipette medium in the plate according to the desired plate layout (for examples, see Tables 9 and 10):
    - Pipette 30 µL medium in all wells of an U-shaped 96-well plate
    - Add 30 µL extra medium to the cell control (CC) wells
  - Pipette 30 µL 1:5 serum dilution *in Duplo* in A1-A10 (or A1-A12 for 6 samples) and resuspend well
  - Remove 15 µL of the serum suspension from A1-A10 (or A1-A12 for 6 samples) and discard.
  - Now, there is 45 µL suspension left in the top row of the plate
  - Pipette 15 µL from A1-A10 to B1-B10 (or A1-A12 to B1-B12 for 6 samples) using a multichannel pipette and resuspend well
  - Repeat step 5 for each row until the last dilution is reached; then remove 15 µL from the last dilution row (H1-H10 for 5 samples, G1-12 for 6 samples) and discard

**Table 9. Pipetting scheme for medium in U-bottom plates for plate layout 5 samples.**

	1	2	3	4	5	6	7	8	9	10	11	12
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		VC	CC
A	30	30	30	30	30	30	30	30	30	30	30	60
B	30	30	30	30	30	30	30	30	30	30	30	60
C	30	30	30	30	30	30	30	30	30	30	30	60
D	30	30	30	30	30	30	30	30	30	30	30	60
E	30	30	30	30	30	30	30	30	30	30	30	60
F	30	30	30	30	30	30	30	30	30	30	30	60
G	30	30	30	30	30	30	30	30	30	30	30	60
H	30	30	30	30	30	30	30	30	30	30	30	60

CC = cell control, VC = Virus control

**Table 10. Pipetting scheme for medium U-bottom plates for plate layout 6 samples.**

		1	2	3	4	5	6	7	8	9	10	11	12
		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
10	A	30	30	30	30	30	30	30	30	30	30	30	30
30	B	30	30	30	30	30	30	30	30	30	30	30	30
90	C	30	30	30	30	30	30	30	30	30	30	30	30
270	D	30	30	30	30	30	30	30	30	30	30	30	30
810	E	30	30	30	30	30	30	30	30	30	30	30	30
2430	F	30	30	30	30	30	30	30	30	30	30	30	30
7290	G	30	30	30	30	30	30	30	30	30	30	30	30
VC/CC	H	30	30	30	30	30	30	60	60	60	60	60	60

CC = cell control, VC = Virus control

- Preparation of virus dilutions for VNT and back titration
  - To acquire an end concentration of 100 TCID<sub>50</sub> per well, dilute the virus stock to 120 TCID<sub>50</sub> per 30 µL. Therefore it is often necessary to make predilutions.
  - Prepare the vials needed and add the required amount of medium.

Comment: A minimum of 10 µL of virus stock or predilution should be pipetted, and it is preferred to use two predilutions to minimise the chance of pipetting aberrations

- For the back titration preparation, pipette medium in one sterile 96-wells U-bottom plate according to Table 11:
  - Add 180 µL medium to rows B-H

**Table 11. Pipetting scheme medium back titration dilution plate (U-bottom).**

	TCID <sub>50</sub>	1	2	3	4	5	6	7	8	9	10	11	12
A	<b>100</b>	180	180	180	180	180	180	180	180	180	180	180	180
B	<b>10</b>	180	180	180	180	180	180	180	180	180	180	180	180
C	<b>1</b>	180	180	180	180	180	180	180	180	180	180	180	180
D	<b>0.1</b>	180	180	180	180	180	180	180	180	180	180	180	180
E	<b>0.01</b>	180	180	180	180	180	180	180	180	180	180	180	180
F	<b>0.001</b>	180	180	180	180	180	180	180	180	180	180	180	180
G	<b>0.0001</b>	180	180	180	180	180	180	180	180	180	180	180	180
H	<b>CC</b>	180	180	180	180	180	180	180	180	180	180	180	180

CC = cell control

## Day 0: VNT and back titration (BSL3)

- Virus Neutralisation Test (VNT)
- Prepare the virus suspension in medium (use the virus predilutions as described above in 'Preparation of virus dilutions for VNT and back titration'. In the case it is **more than three months ago** since a back titration was performed, a new virus stock titration must be done prior to the experiment to redefine the virus titer
  - Add 30 µL (120 TCID<sub>50</sub>) virus suspension to all wells of the plates with serum dilutions, except for the cell control (CC) wells;
  - Incubate for one hour at 35 °C and 5% CO<sub>2</sub>;
  - During incubation, prepare the cell plates at BSL2. Carefully remove medium from 96-wells F-bottom plates with Vero-E6 cells by using the Serres system;
  - Replace the DMEM (5%) medium with 100 µL pre-warmed DMEM (2%);
  - In the BSL3 lab, carefully transfer 50 µL of the serum-virus mixture from the U-Bottom dilution plates to the F-bottom plates containing the cell monolayer. The final volume in each of the F-Bottom plates is 150 µL;
  - Incubate for 48 hours at 35 °C and 5% CO<sub>2</sub>;
- Virus titer check (back titrations, see tables 3 and 4)
  - Pipette 180 µL of the virus suspension (120 TCID<sub>50</sub> / 30 µL) in row A of the 96-wells U-bottom virus dilution plate (Table 3);
  - Take new pipette tips and transfer 20 µL from row A to row B and resuspend well. Discard tips;
  - Repeat step 2 until row G is reached and remove 20 µL from row G;
  - Incubate for one hour at 35 °C and 5% CO<sub>2</sub>;
  - During incubation, prepare the cell plates at BSL2. Carefully remove medium from 96-wells F-bottom plates with Vero-E6 cells by using the Serres system;
  - Replace the DMEM medium (5%) with 125 µL pre-warmed DMEM (2%);

- In the BSL3 lab, carefully transfer 25 µL of the virus dilutions from the U-Bottom dilution plates to the F-bottom plates containing the cell monolayer. The final volume in each of the F-Bottom plates is 150 µL;
- Incubate for 48 hours at 35 °C and 5% CO<sub>2</sub>.

### Optional: Virus stock titration (once per 3 months) (BSL3)

- Virus stock titration
  - Take a sterile 96-wells U-bottom dilution plate and pipette 180 µL medium in columns 1-12 (Table 12);
  - Pipette 20 µL of the virus stock (undiluted) in all wells of column 1 of the U-bottom dilution plate and resuspend. Since this is a 1:10 dilution, at least 10 times should be enough, preferably more. Take a new pipette tip for each well;
  - Using a multichannel, transfer 20 µL from column 1 to column 2 and resuspend well. Discard tips.
  - Repeat step 4 until column 11 is reached;
  - Incubate for one hour at 35 °C and 5% CO<sub>2</sub>;
  - Carefully remove medium from 96-wells F-bottom plates with Vero-E6 cells by using the Serres system. Add 125 µL fresh DMEM (2%) to all wells of the plates;
  - In BSL3 lab, carefully transfer 25 µL from the wells of the U-bottom virus dilution plate to the corresponding wells of the 96-wells F-bottom plate. The final volume in the F-bottom plate is 150 µL;
  - Incubate for 48 hours at 35 °C and 5% CO<sub>2</sub>.

**Table 12. Pipetting scheme medium Virus stock titration dilution plate (U-bottom)**

	1	2	3	4	5	6	7	8	9	10	11	12
	<b>10<sup>-1</sup></b>	<b>10<sup>-2</sup></b>	<b>10<sup>-3</sup></b>	<b>10<sup>-4</sup></b>	<b>10<sup>-5</sup></b>	<b>10<sup>-6</sup></b>	<b>10<sup>-7</sup></b>	<b>10<sup>-8</sup></b>	<b>10<sup>-9</sup></b>	<b>10<sup>-10</sup></b>	<b>10<sup>-11</sup></b>	<b>CC</b>
A	180	180	180	180	180	180	180	180	180	180	180	180
B	180	180	180	180	180	180	180	180	180	180	180	180
C	180	180	180	180	180	180	180	180	180	180	180	180
D	180	180	180	180	180	180	180	180	180	180	180	180
E	180	180	180	180	180	180	180	180	180	180	180	180
F	180	180	180	180	180	180	180	180	180	180	180	180
G	180	180	180	180	180	180	180	180	180	180	180	180
H	180	180	180	180	180	180	180	180	180	180	180	180

CC = cell control

#### Day +2: Fixation (BSL3)

- Fixation
  - Check the back titration plate after 48 hours under the microscope to ensure that there is CPE in row A and a monolayer in row H;
  - Some variants show no or little CPE;
  - Fixate cells with 150 µL/well 10% paraformaldehyde in PBS (leave the medium in the wells!) for at least 45 minutes;
  - If plates are not stained the same day, cover the plate with aluminum foil and incubate in the fridge at 2-8 °C;
  - Remove fixation buffer from the wells by emptying the plates in a container with a small layer of 70% ethanol inside;
  - Next, take another container filled with a layer 70% ethanol (sufficient to immerse the plates), and immerse the plates and their covers (separately) in 70% ethanol. Make sure that every surface has been in contact with 70% ethanol;
  - Empty the plates in the container and transport them from the BSL3 while ethanol is still vaporising.

Comment: If the staining cannot take place on the same day, it is best to leave the plates on the BSL3 lab in the 10% paraformaldehyde fixation buffer in the fridge. This can be stored for up to seven days. In the case the plates necessarily must be removed from the BSL3 but staining can only be done the next day, they should be transported from the BSL3 after completing the steps described in 'Fixation'. In the BSL2 lab, fill all wells of the plates with 100 µL 1x PBS. Cover the plates with their covers, put the plates in a biocarrier and store them in the fridge on BSL2 at 2-8 °C. The plates can be stored in 1x PBS for a maximum of 24 hours.

#### Day +2 - +9: Staining (BSL2)

- Preparation
  - Make sufficient buffers and solutions for the amount of plates that need to be stained. Make sure that there is sufficient PBS in storage and that any open PBS flasks are not contaminated;
- Staining

- Wash all plates 1x with PBS (300 µL/well) using the BioTek washer (program “VNT”);
- Add 100 µL/well permeabilization buffer.
- Incubate for 10 minutes at RT;
- Wash all plates 2x with PBS (300 µL/well) using the BioTek washer (program “VNT 2x”);
- Dilute the primary antibody (with a concentration of 1,1mg/ml) 1:5 000 in dilution buffer (2 µL primary antibody per 10 mL dilution buffer). Add 100 µL/well;
  - Note the batch number.
- Incubate for one hour at RT;
- Wash all plates 3x with PBS (300 µL/well) using the BioTek washer (program “VNT 3x”);
- Dilute the secondary antibody 1:15.000 in dilution buffer (1 µL secondary antibody per 15 mL dilution buffer). Add 100 µL/well;
  - Note the batch/LOT number.
- Incubate for one hour at RT. In the meantime, equilibrate the TMB substrate to RT (at least 15 minutes);
- Wash all plates 3x with PBS (300 µL/well) using the BioTek washer (program “VNT 3x”);
- Add 100 µL TMB substrate to the wells and incubate 15-20 minutes at RT until the cell control (CC) starts to color blue. Make sure that every plate incubates the same amount of time;
- Stop the coloring reaction by adding 100 µL 0.5M H<sub>2</sub>SO<sub>4</sub> to all wells; the color will change from blue to yellow;
- Read the plates at 450/620 nm using the plate reader and MikroWin software.

Comment: also export the data as a .txt file

## Data analyses

### Determining the virus titer and the average values of cell and virus controls

- Open the .txt file; replace all the commas by a dot.
- Open the F030a: 'F030a Analysis titers VNT SARS-CoV-2' file.
- Always start the analysis by determining the virus titer (first tab in F030a).
  - Copy the data from the .txt to the Excel file, and all the data will be processed. First, 2x cell control (CC; row H) will be subtracted from the data values. Then, the positive wells will be scored with '1' and the negative wells with '0'. The titer that follows is the back titration value and indicates how much virus was added to the wells. A value of 100 is ideal, but anywhere between 50 and 200 is satisfactory to analyse the samples.
- Next, fill in the sample numbers or codes. This must be done per plate, so make sure to check the pipetting schemes for the correct sample loading.
- For analysis of sample titers, there is a separate tab (third tab for five samples, fourth tab for six samples). Copy the data from the .TXT file to Excel. The average of virus control and cell control is calculated automatically. You will need these two values for further analysis in GraphPad.

### Analyse data in GraphPad from scratch using the following steps

- Copy data to GraphPad. Select 'New table & graph' > 'XY' > 'Options' > 'X: Numbers' and 'Y: Enter 2 replicate values in side-by-side columns'. Press 'Create'.
- Under 'X' fill in the dilutions: 10; 30; 90; 270; 810; 2.430; 7.290(; 21.870).
- Copy data of the samples from plate 1 (column 1-10 or columns 1-12; rows A-G) from Excel to GraphPad, depending on the plate layout.
- Change the name under 'Data tables' to 'Plate 1'. All the linked data will be renamed to Plate 1 as well.
- Creating an S-curve. To view the graphs, press 'Plate 1' under 'Graphs'. You can now select to plot 'Points only' graph (without a line), with a Mean and Error. This should be the standard setting.
- To convert the sera dilutions on X-axis to a log scale: select 'Analyse' in the top menu. Then select 'Transform'. Turn on 'Transform X values using X=Log(X)'.
- To normalise the graph with virus control = 100%, and the cell control = 0%, press 'Analyse' > 'Normalise' again
  - Under 'How is 0% defined?' you need to enter Y = the average value of the cell control as calculated in the F030a file for that specific plate.
  - Under 'How is 100% defined?' you need to enter Y = the average value of the virus control, which can be found in the same Excel file.
- To create an S-curve and calculate the IC<sub>50</sub>, go to 'Analyse' > 'XY analyses' > 'Nonlinear regression (curve fit)'. Choose 'Dose-response - inhibition' > '(log)inhibitor vs response - variable slope (four parameters)'.
- Under the tab 'Constrain' > 'Bottom' > 'Must be greater than 0' and 'Top' > 'Must be less than 100'.

- Evaluation graphs and titers. Under 'Results' > 'Nonlin fit of Normalize of Transform of plate 1' you will find the titers at 'IC50'. Copy these values to the Excel file.

Comment: It is very important that you check the graphs to see whether the calculated titer is realistic before copying the values to Excel.

- To check the graphs manually, you need to go back to the graphs. To make them clearer, you can adjust the following settings:
  - In the top menu click 'Change' > 'Change colours' > 'Colours'. Every sample will be assigned a different colour
  - Mark the sera dilutions on the X-axis: double click on the X-axis > 'Number format' > 'Antilog'.
  - Add a line at 50% (for IC): go to 'Left Y axis' (or double click on the Y-axis) > 'Additional ticks and grid lines Y=50' > select 'line'.
  - Adjust the Y-axis label to 'Relative infection (%)'.
- Check for every graph whether the calculated titer is correct (realistic). Adjust it to <10 for negative results, and to >21.780 or >7.290 (depending on the maximum dilution you have tested) for maximum results. In case of an obvious outlier you can ignore these values under 'Data Tables'. Right click on the outlier value and click on 'Exclude values'. Report a reason why you ignore a value.
- For the next plate: delete unnecessary graphs. Under 'Graphs' delete both 'Plate 1' and 'Transform of Plate 1'.
- Under 'Data Tables', select 'Plate 1' and right click on 'Duplicate Family'. Make sure that 'Also duplicate linked sheets' is checked and name the next plate Plate 2 under 'New sheet name' (or the plate number you were). Everything will be copied. Delete the values under Data Tables > Plate 2.
- Copy the values of the next plate from Excel to GraphPad (in the new family you just created). To adjust the parameters of the normalization, select the graph and click on the red raster icon ('Change analysis parameters') next to the 'Analyse' button > 'Change parameters of normalise of transform of Plate 2'. Adjust the 0%Y and 100%Y values to those of the current plate.
- Copy the IC<sub>50</sub>-value under 'Results' > 'Nonlin fit of Normalise of transform of Plate 2' to. Check manually whether the titers are correct
- Repeat from until all the plates have been analysed.

## 2.7 Antibody-based detection methods

### 2.7.1 Immunofluorescence assay (IFA)

#### *Introduction*

Cells seeded on round glass slides that fit into a 24-well plate are infected with SARS-CoV-2. After two days, the cells are stained with anti-SARS-CoV-2 nucleocapsid antibody which is visualised with a fluorescent second antibody.

#### *Materials and reagents*

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- sterile 24 µm filter;
- round glass slides that fit into 24-well plate;
- PBS;
- 80% acetone;
- SARS-CoV Nucleocapsid Antibody, Rabbit Pab, Sino Biological;
- Secondary antibody: Alexa Fluor 488-F(ab')<sub>2</sub> fragment of goat anti-rabbit igG (H+L), Invitrogen;
- Vero-E6 cells;
- Calu-3 cells;
- fluid from PCR-positive swab.

#### *Method*

##### **Preparation of cells**

- Vero-E6 cells (used for pre-VOC isolates) are seeded one day in advance: split ratio 1:10 from 90% confluent flask.
- Calu-3 cells (for VOC isolates) are seeded six days in advance: split ratio 1:10 from 90% confluent flask.

**Day 0**

- Filter the fluid from a PCR-positive swab (from the anterior nose or the nasopharynx) through a 24 µm filter.
- Wash monolayer of cells seeded on round glass slides in a 24-well plate with PBS.
- Add 1 mL medium to the cells.
- Add 100 µL filtered swab fluid.
- Centrifuge the plate for 10 minutes at 350xg.
- Incubate the plate at 37 °C in a CO<sub>2</sub> incubator.

**Day 2**

- Wash the cells once with PBS.
- Take the glass slide out of the well and fix in 80% acetone for 30 minutes.
- Fix the round glass on a regular microscope slide.
- Evaluate under a fluorescent microscope.

## 2.8 Pseudovirus assays

### 2.8.1 VSV pseudovirus production

#### *Introduction*

The protocol for VSV-G PP (Vesicular stomatitis virus G glycoprotein pseudoparticles) rescue was adapted from the references [27] and [28].

For more information see: references [27] and [28].

For this protocol, the plates are scanned using an Amersham Typhoon laser scanner. Individual infected cells are quantified using the ImageQuant TL analysis software.

VSV rescue plasmids ordered from the Addgene repository:

- pVSV-eGFP-dG (#31842)
- pMD2.G (#12259)
- pCAG-VSV-P (#64088)
- pCAG-VSV-L (#64085)
- pCAG-VSV-N (#64087)
- pCAGGS-T7Opt (#65974)

#### *Materials and reagents*

- PIPETBOY;
- serological pipettes;
- pipettes;
- filter tips;
- 96-well plate;
- plasmids;
- PEI 1 mg/mL transfection reagent;
- Opti-MEM I (1×) + GlutaMAX;
- HEK-293T cells;
- Vero-E6 cells;
- culture medium.

#### *Method*

- Prepare a 70% confluent 10 cm dish of HEK-293T cells.
- Transfect the cells with 10 µg pVSV-eGFP-dG, 2 µg pCAG-VSV-N (nucleocapsid), 2 µg pCAG-VSV-L (polymerase), 2 µg pMD2.G (glycoprotein, VSV-G), 2 µg pCAG-VSV-P (phosphoprotein), and 2 µg pCAGGS-T7Opt (T7 RNA polymerase) using PEI at a ratio of 1:3 (DNA:PEI) in Opti-MEM I (1×) + GlutaMAX.
- Transfer the supernatant 48 hours post-transfection onto new plates transfected 24 hours prior with VSV-G.
- Re-transfect these plates after a further 48 hours with VSV-G.
- Collect resulting PPs after 24 hours.
- Clear PPs by centrifugation at 2000×g for five minutes.
- Store PPs at -80 °C.

#### **Subsequent VSV-G PP batch production**

- Infect the VSV-G transfected HEK-293T cells with VSV-G PPs at an MOI of 0.1.

### Titre determination

- Prepare 10-fold serial dilutions in Opti-MEM I (1×) + GlutaMAX.
- Add aliquots of each dilution to monolayers of  $2 \times 10^4$  Vero-E6 cells in the same medium in a 96-well plate.
- Perform three replicates per PP stock.
- Incubate the plates at 37 °C overnight.
- Scan the plates using an Amersham Typhoon laser scanner.
- Quantify individual infected cells using ImageQuant TL analysis software.

## 2.8.2 Spike expressing pseudovirus production (from in-house SOP)

### Introduction

Spike-expressing pseudoviruses are a powerful tool for studying virus entry that would normally require BSL-3 or BSL-4 containments. As a result, these studies are also feasible for laboratories that do not have access to BSL-3 or BSL-4 facilities. In those pseudoviruses, the glycoprotein gene is removed and replaced with a fluorescent reporter gene, such as green fluorescent protein (GFP). This allows accurate titres of the pseudotyped virus to be determined by counting individual cells infected with the pseudotypes.

### Materials and reagents

- HEK-293T cell culture media;
- Calu-3 medium;
- Opti-MEM I (1×) + GlutaMAX (both with and without Pen/Strep);
- trypsin;
- PBS;
- PEI 1 mg/mL transfection reagent;
- High quality maxiprep of Spike Pseudotyping Vector, InvivoGen;
- High quality VSV- $\Delta$ G-GFP virus pseudotyped with VSV glycoprotein stock with a known titre;
- Anti-VSV antibody (Kerafast Cat#EB0010)
  - 1:50 000 in Opti-MEM + Pen/Strep.

### Method

#### Plating 293T cells (Day-1)

- Pre-warm 293T cell culture medium and trypsin in a 37 °C water bath.
- Harvest the 70% confluent 293T cells by trypsinisation (1 mL/plate).
- Plate the 293T cells 1:3 in 10 cm dishes.
- Incubate in a 37 °C CO<sub>2</sub> incubator.

#### Transfection (Day 0)

- To prepare transfection mix (DNA to PEI ratio is 1:3):
  - Add 15 µg/dish Spike plasmid to 1 mL/dish Opti-MEM without Pen/Strep, mix add 45 µg PEI and mix well, leave it for 30 minutes at RT.
  - Refresh the 293T cells with 9 mL/dish of Opti-MEM with Pen/Strep.
  - Add 1 mL/dish of transfection mix dropwise.
  - Place in a 37 °C CO<sub>2</sub> incubator.

#### Infection (Day 1) and harvest (Days 2 and 3)

- Replace the medium for fresh OptiMEM with Pen/Strep containing approximately 1-3 MOI VSV- $\Delta$ G pseudoparticles pseudotyped with VSV glycoprotein.
- After four hours, wash the plates thrice with 5 mL warm Opti-MEM + Pen/Strep, and replace with 10 mL Opti-MEM + PenStrepPen/Strep50 000 anti-VSV antibody.
- Check GFP expression after 24 hours with a fluorescence microscope (acceptable if >50% cells are positive).
- Collect the medium at 24 hours and 48 hours post-infection, and spin down twice at 2 000xg for five minutes to remove cell debris.
- Keep at 4 °C for up to one week before discarding.
- Titrate as for VSV pseudovirus production on 100% confluent Calu-3 cells.

## 2.8.3 Pseudovirus neutralisation using the Opera Phenix spinning disk confocal HCS system

### *Introduction*

The protocol for pseudovirus neutralisation was adapted from the reference [27].

### *Materials and reagents*

- pipettes;
- filter tips;
- 96-well V-bottom plates;
- PBS;
- paraformaldehyde;
- Hoechst dye;
- sera;
- Opti-MEM I (1×) + GlutaMAX (Gibco);
- Calu-3 cells;
- pseudovirus.

### *Method*

- Heat inactivate sera for 30 minutes at 56 °C.
- Dilute the sera two-fold serially in 60 µL of Opti-MEM I (1×) + GlutaMAX (Gibco) in 96-well V-bottom plates.
- Add 400 PFU in 60 µL per well to a final volume of 120 µL and a serum dilution of 1:20 in the first well.
- Incubate plates for one hour at 37 °C.
- Add 100 µL of pseudovirus and serum mix to confluent monolayers of Calu-3 cells.
- Incubate the infected plates overnight in a 37 °C CO<sub>2</sub> incubator.
- Fix the plates in paraformaldehyde and wash in PBS.
- Stain the nuclei with Hoechst dye for 30 minutes.
- Image the cells using the Opera Phenix spinning disk confocal HCS system.
- Quantify the number of GFP-positive/Alexa Fluor 488-positive infected cells using the Harmony high-content imaging and analysis software.
- Calculate PRNT<sub>50</sub> on the basis of nonlinear regression, followed by a Pearson correlation and linear regression analysis.

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