ecdc
EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

TECHNICAL REPORT

Standard laboratory protocols for SARS-CoV-2 characterisation

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

Standard laboratory protocols for SARS-CoV-2 characterisation

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC) [reference: Work Package 3 Diagnostic and virus characterisation support, of the ECDC project, NP/2021/DPR/25121]. It was coordinated by Eeva Broberg and Annette Kraus, and produced by the AURORAE consortium.

Authors
The AURORAE consortium:

- Adam Meijer, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
- Christian Drosten, Charité - Universitätsmedizin Berlin, Berlin, Germany
- Ron A.M. Fouchier, Erasmus University Medical Center, Rotterdam, the Netherlands
- Bart L. Haagmans, Erasmus University Medical Center, Rotterdam, the Netherlands
- Victor Corman, Charité - Universitätsmedizin Berlin, Berlin, Germany
- Kim Heimsch, Charité - Universitätsmedizin Berlin, Berlin, Germany
- Bas Oude Munnink, Erasmus University Medical Center, Rotterdam, the Netherlands
- Babs E. Venestrepen, Erasmus University Medical Center, Rotterdam, the Netherlands
- Ramona Mögling, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
- Marc Soeteman, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Acknowledgments
We thank the whole AURORAE consortium for their support and the provision of protocols. We also thank the reviewers Angeliki Melidou (ECDC), and Kevin Bewley, Yann Le Duff, and Naomi Coombes (United Kingdom Health Security Agency, London, United Kingdom) for their valuable comments.

Suggested citation: European Centre for Disease Prevention and Control. Standard laboratory protocols for SARS-CoV-2 characterisation. Stockholm: ECDC; 2023.

Stockholm, October 2023

ISBN 978-92-9498-656-6
doi: 10.2900/209861
Catalogue number TQ-04-23-928-EN-N

Contents

Tables

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
$NaHOC_3$	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
$PRNT_{50}$	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)
$TCID_{50}$	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 overview of commonly used laboratory protocols for the characterisation of SARS-CoV-2 and influenza viruses, 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.

1 Background

1.1 General background

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) resulted in a worldwide pandemic of coronavirus disease 2019 (COVID-19), which led to over 750 million confirmed cases worldwide, and over 6.5 million deaths (as of 7 March 2023) [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, 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 requires different laboratory safety levels.

Culturing 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 Culturing of cells

Introduction

In this section, the culturing 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 be routinely tested for mycoplasma and parainfluenza virus 5 (PIV5) (see sections 2.1.3, 2.1.4, and 2.1.5).

The composition of the media may vary depending on the laboratory. Therefore, several options are provided here. Once a culturing medium has been chosen, ideally it should 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, $6x10^5$ cells/mL (= $3x10^5$ 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 $NaHCO_3$ 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: Culturing 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 Pen/Strep (10 000 U/mL; 10 000 µg/mL; Lonza, 17-602E).

or DMEM supplemented with 10% FBS only.

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

Infection medium

Advanced DMEM/F-12 (without $NaHCO_3$ 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-E6 cells

ATCC number: CRL-1586 [13]

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

Comment: When plates are seeded for an experiment, $3x10^5$ cells/mL (=$1.5x10^5$ 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

- 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: Culturing 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).

or DMEM supplemented with 10% FBS only.

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

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).

Vero-hSLAM and Vero-TMPRSS2 can be cultured in the same way, however, addition of 0.4 mg/mL geneticin needs to be added to the medium.

HEK-293T

ATCC number: CRL-3216 [14]

A sub-culturing 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
RFA	6.6 µL
Eluate	8.0 µL
Total	**20.0 µL**

**TFA, Thermo Fisher Scientiifc 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
MgCl2 (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
RFA	29.5 µL
Total	**50.0 µL**

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	GCGGTTCCAAACGTACCAA	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	TGCACCACCTGTCATTGGGTTGACCTC	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
- RFA.

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
Total	**12.5 µL**

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);
- FBS
- freezing medium: 80% FBS + 20% DMSO.

Method

- Approximately, $1x10^6$ - $1x10^7$ 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, 5 minutes, RT).
- Cell pellet is resuspended in 0.5 mL FBS.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
- CO_2 incubator.

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 $3x10^5$ 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_2 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_2 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_2 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_2 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, $TCID_{50}$ 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 $3x10^5$ 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

CO_2 incubator.

Method

Stock production

- Vero-E6 cells (culturing 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 µL 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_2.

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 µL 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 µL 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 $TCID_{50}$

Introduction

This protocol describes the method to determine the $TCID_{50}$ of SARS-CoV-2.

SARS-CoV-2 is isolated from positive clinical specimens for various purposes. Vero-E6 cells (seed $3x10^5$ 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;
- 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
- CO_2 incubator.

Method

Preparation of cell plates

- Prepare cells ahead of time, dependenting 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 $TCID_{50}$ based on the Spearman–Karber formula [19].

2.3.2 Titrating stock for $PRNT_{50}$ 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
- CO_2 incubator.

Method

Preparation of cell plates

- Prepare cells ahead of time, dependenting 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^2; maximum spot size: 9.6296 mm^2.

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 [20].

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)
- RFA.

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
RFA	6.6 µL
RNA	8.0 µL
Total	**20.0 µL**

**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	CARATGTTAAASACACTATTAGCATA	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

2.5.2 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 [40 U/µL]	0.5 µL
RNA template	10 µL
Total	12.5 µL

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
Total	20 µL

PCR amplification

For the PCR amplification, the primers of ARCTIC version 4.1 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
RFA	15.55 µL
Total	22.5 µL
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 room temperature for 30 minutes.
- Vortex the beads for 10 seconds.
- Add 40 µL beads to 50 µL sample (add 10 µL 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 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 samples on the magnet and add 200 µL of 80% ethanol.
- Incubate at RT for ≥30 seconds.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples to dry at RT for ~30 seconds, or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 53 µL 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 µL 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 µL (if the concentration is too low, use 52 µL and add 1.5 µL water).

Library preparation (SQL-LSK109, Oxford Nanopore protocol)

DNA repair and end-prep

Reagent	Volume
DNA	53.5 µL
Ultra II End Prep reaction buffer	3.5 µL
Ultra II End Prep enzyme mix	3.0 µL
Total	**60 µL**

PCR machine: Five minutes at 20 °C, and five minutes at 65 °C.

- Add 60 µL 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 µL 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 µL of 80% ethanol.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples to dry at RT for ~30 seconds, or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 25 µL 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 µL 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 µL.

Native barcode ligation

Reagent	Volume
500 ng end-prepped DNA	22.5 µL
Native barcode	2.5 µL
Blunt/TA Ligase Master Mix	25 µL
Total	**50 µL**

Mix by pipetting and incubating for 10 minutes at RT.

- Add 50 µL 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 µL 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 µL of 80% ethanol.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples 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 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 µL 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 µL (for 48 samples: 7.3 ng/sample, for 96 samples: 3.65 ng/sample). If the pooled sample volume exceeds 65 µL, add the same volume of AMPure XP beads, elution buffer in 65 µL.

Adapter ligation and clean-up

Thaw one tube of Short Fragment Buffer (SFB).

Reagent	Volume
700 ng pooled barcoded sample DNA	65 µL
Adapter Mix II Expansion(AMII)	5 µL
NEBNext Quick Ligation Reaction Buffer (5x)	20 µL
Quick T4 DNA Ligase	10 µL
Total	**100 µL**

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

- Add 80 µL 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 µL 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 µL SFB.
- Spin down the samples and pipette off any residual supernatant.
- Allow the samples to dry at RT for ~30 seconds, or until the beads are dry.
- Remove the samples from the magnet.
- Resuspend the DNA using 15 µL 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 ~15 µL of the supernatant and transfer it to a new DNA LoBind tube.

Preparation to load the flow cell[i]

Store the reagents on ice.

Flow cell priming was prepared:

Reagent	One reaction (µL)
FLT	30 µL
1 tube Flush Buffer (FB)	volume not known

The library was prepared for loading:

Reagent	One reaction (µL)
Sequencing Buffer (SQB)	45.5 µL
Loading Beads (LB) (mix before adding)	25.5 µL
DNA library (50 ng)	~4 µL
Total	**75 µL**

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.

[i] Flow cell priming mix was prepared.

Check the Oxford Nanopore Technologies website to keep the protocol updated.

- 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
- CO_2 incubator.

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_2.
- Incubate serum/virus samples on seeded Vero-E6 cells for one hour at 37 °C and 5% CO_2.
- Overlay cells with 1.2% Avicel/DMEM for three days at 37 °C and 5% CO_2.
- 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 ($1.5x10^5$ cells/24-well).
- Incubate serum+virus samples (200 µL) on the cells for one hour at 37 °C and 5% CO_2.
- 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_2.
- 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 $FRNT_{50}$ (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
- CO_2 incubator.

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 $5.0x10^5$ cells/mL (Vero-E6) or $4.0x10^5$ (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 $PRNT_{50}$).
- 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 µL; ctrl : control; black: volume for maintenance medium; blue: volume for serum

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

- Add 12 µL of serum to designated wells (blue in the schedule above).
- Transfer 60 µL from row A to row B and resuspend at least eight times.
- Change the pipette tips.
- Transfer 60 µL from row B to row C and resuspend at least eight times.
- Change the pipette tips.
- Repeat until row H and discard 60 µL from row H.
- The final volume should be 60 µL 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 µL 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 µL.
- 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 µL 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 µL 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 µL/well 0.5% Triton X-100-PBS.
- 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 peroxidase substrate (e.g. TrueBlue).
- Incubate for 5–30 minutes in the dark at room temperature.
- 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^2; maximum spot size: 9.6296 mm^2.

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 $PRNT_{50}$ 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 - $2x10^5$ cells/mL)
- CO_2 incubator.

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 $TCID_{50}$ 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_2.

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

- Add 100 µL cell suspension per well for the whole plate.
- Per well 1.8 –$2x10^4$ cells are needed
  - 1.8 – $2x10^5$ cells/mL
  - 1.8 – $2x10^6$ cells/plate (= 10 mL)
- Incubate for three days at 35 °C and 5% CO_2.

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

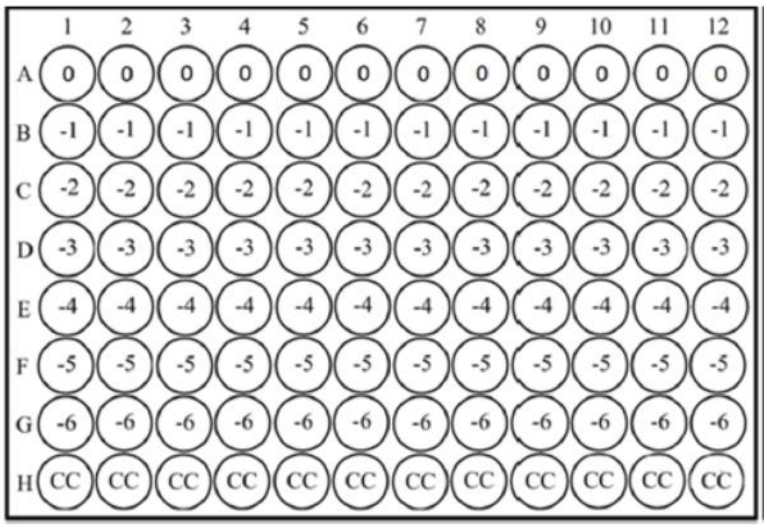
- Pre-warm the Vero-E6 medium.
- Add 25 µL Vero-E6 medium to a 96-well plate (flat-bottom) in rows A to G.
- Add 50 µL Vero-E6 medium to a 96-well plate (flat-bottom) in row H (CC).
- Take a sterile 96-well-plate (U-bottom) for dilutions and add 180 µL Vero-E6 medium to the wells of rows B to H.
- Pipette 180 µL of the virus dilution that was prepared for the VNT (100 $TCID_{50}$/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_2.

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

- Add 100 µL cell suspension per well
  - Per well 1.8 - $2x10^4$ cells needed
    - 1.8 - $2x10^5$ cells/mL
    - 1.8 - $2x10^6$ cells/plate (= 10 mL)
- Incubate for three days at 35 °C and 5% CO_2.

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_{90}: a well is positive (1) if less than 90% of the cell layer is intact (≥ 1 plaque(s)).
- VNT_{50}: 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 $TCID_{50}$/25 µL.
- Note: for a back titration to be exactly 100 $TCID_{50}$ 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.

2.7 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')2 fragment of goat anti-rabbit igG (H+L), Invitrogen;
- Vero-E6 cells;
- Calu-3 cells;
- fluid from PCR-positive swab
- CO_2 incubator.

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_2 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 [24] and [25].

For more information see: references [24] and [25].

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
- CO_2 incubator.

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 2 000×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 2×10^4 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-ΔG-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_2 incubator.

Transfection (Day 0)

To prepare transfection mix (DNA to PEI ration 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_2 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-ΔG 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 [24].

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_2 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 $PRNT_{50}$ on the basis of nonlinear regression, followed by a Pearson correlation and linear regression analysis.

References

1. WHO coronavirus (COVID-19) dashboard. As of March 10, 2023. Last retrieval March 10, 2023. Online at: https://covid19.who.int.
2. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. Nat Rev Mol Cell Biol. 2022;23:3-20. Doi: https://doi.org/10.1038/s41580-021-00418-x.
3. Chinta S, Rodriguez-Guerra M, Shaban M, Pandey N, Jaquez-Duran M, Vittorio TJ. COVID-19 therapy and vaccination: a clinical narrative review. Drugs Context. 2023;12:2022-7-2. Doi: https://doi.org/10.7573/dic.2022-7-2.
4. Benedetti C, Waldman M, Zaza G, Riella LV, Cravedi P. COVID-19 and the Kidenys: An Update. Front. Med. 2020; 7:423. Doi: 13.3389/fmed.2020.00423.
5. Lamers MM, Haagmanns BL. SARS-CoV-2 pathogenesis. Nat Rev Microbiol. 2022; 20(5):270-284. Doi: 10.1038/s41579-022-00713-0.
6. Aguilar-Bretones M, Fouchier RA, Koopmans MP, van Nierop GP. Impact of antigenic evolution and original antigenic sin on SARS-CoV-2 immunity. 2023; 133(1):e162192. Doi: 10.1172/JCI162192.
7. Plante JA, Mitchell BM, Plante KS, Debbrik K, Weaver SC, Menachery VD. The variant gambit: COVID-19's next move. Cell Host Microbe. 2021; 29(4):508-515. Doi: 10.1016/j.chom.2021.02.020.
8. Mykytyn AZ, Rosu ME, Kok A, Rissmann M, van Amerongen G, Geurtsvankessel C, de Vries RD, Oude Munnink BB, Smith DJ, Koopmans MPG, Lamers MM, Fouchier RAM, Haagmans BL. Antigenic mapping of emerging SARS-CoV-2 omicron variants BM.1.1.1, BQ.1.1, and XBB.1. Lancet Microbe. 2023; S2666-2547(22)00384-6. Doi: 10.1016/S2666-5247(22)00384-6.
9. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DGJC, Haagmans BL, Van der Veer, Van den Brink S, Wijsman L, Goderski G, Romette J-L, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MPG, Drosten C. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020 Jan;25(3):2000045. Doi: https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045.
10. Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC) [internet]. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32000L0054#document1 .
11. Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs). Licence: CC BY-NC-SA 3.0 IGO. Available from: https://osha.europa.eu/en/legislation/directives/exposure-to-biological-agents/77
12. American tissue culture collection (ATCC) HTB-55 [internet]. Available from: https://www.atcc.org/products/htb-55.
13. American tissue culture collection (ATCC) CRL-1586 [internet]. Available from: https://www.atcc.org/products/crl-1586 Available from: .https://www.atcc.org/products/crl-1586
14. American tissue culture collection (ATCC) CRL-3216 [internet]. Available from: https://www.atcc.org/products/crl-1586
15. Doornum GJJ, Guldemeester J, Osterhaus ADME, Niesters HGM. Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol. 2003 Feb 41(2):576-80. Doi: 10.1128/JCM.41.2.576–580.2003.
16. Kuppeveld JFM, Johansson KE, Galama JMD, Kissing J, Bölske G, Van der Logt JTM, Melchers WJG. Detection of mycoplasma contamination in cell cultures by a mycoplasma group-specific PCR. App Environ Microbiol. 1994 Jan 60(1):149-152. Doi: 10.1128/aem.60.1.149-152.1994.
17. Matsuyama S, Nao N, Shirato K, Takeda M. Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. Proc Natl Aciad Sci U S A. 2020 Mar 31;117(13):7001-7003. Doi: 10.1073/pnas.2002589117.
18. Funnell SGP, Afrough B, Baczenas JJ, Berry N, Bewely KR, Bradford R, Florence C, Le Duff Y, Lewis M, Moriarty RV, Connor SLO, Osman KL, Pullan S, Rashid S, Richards KS, Stemple KJ, Knezevic I. A cautionary perspective regarding the isolation and serial propagation of SARS-CoV-2 in Vero cells. NPJ Vaccines. 2021 Jun 17;6(1):83. Doi: 10.1038/s41541-021-00346-z.
19. Chengfeng Lei, Jian Yang, Jia Hu, Xiulian Sun. On the Calculation of TCID50 for Quantitation of Virus Infectivity .VIROLOGICA SINICA, 2021, 36(1) : 141-144. http://dx.doi.org/10.1007/s12250-020-00230-5 Tool available at: https://www.virosin.org/tcid50/TCID50.html#:~:text=Spearman%E2%80%93K%C3%A4rber%20Method%3A%20Calculate%20now,of%20test%20units%20per%20dilution
20. Hoek RA, Marthe SA, Pas SD, Bakker M, Hoogsteden HC, Boucher CAB, Van der Eerden MM. Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients. Scand J Infect Dis. 2013 Jan;45(1):65-9. Doi: 10.3109/00365548.2012.708942.
21. Herzog P, Drosten C, Müller MA. 2008. Plaque assay for human coronavirus NL63 using human colon carcinoma cells. Virol J 5:138. Doi: 10.1186/1743-422X-5-138.
22. Wolfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller MA, Niemeyer D, Jones TC, Vollmar P, Rothe C, Hoelscher M, Bleicker T, Brunink S, Schneider J, Ehmann R, Zwirglmaier K, Drosten C, Wendtner C. 2020. Virological assessment of hospitalized patients with COVID-2019. Nature. 2020;581(7809):465-469. Doi: 10.1038/s41586-020-2196-x.

23. Bewley KR, Coombes NS, Gagnon L, McInroy L, Baker N, Shaik I, St-Jean JR, St-Amant N, Buttigieg KR, Humphries HE, Godwin KJ, Brunt E, Allen L, Leung S, Brown PJ, Penn EJ, Thomas K, Kulnis G, Hallis B, Carroll M, Funnell S, Charlton S. Quantification of SARS-CoV-2 neutralizing antibody by wild-type plaque reduction neutralization, microneutralization and pseudotyped virus neutralization assays. Nat Protoc. 2021;16(6):3114-3140. Doi: 10.1038/s41596-021-00536-y.

24. Mykytyn AZ, Breugem TI, Riesebosch S, Schipper D, Van den Doel PB, Rottier RJ, Lamers MM, Haagmanns BL. SARS-CoV-2 entry into human airway organoids is serine protease-mediated and facilitated by the multibasic cleavage site. eLife 10:e64508. Doi: https://doi.org/10.7554/eLife.64508 .

25. S P Whelan, L A Ball, J N Barr, and G T Wertz. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. PNAS 92(18):8388-8392. Doi: https://doi.org/10.1073/pnas.92.18.8388 .

European Centre for Disease Prevention and Control (ECDC)

Gustav III:s Boulevard 40
16973 Solna, Sweden

Tel. +46 858601000
ECDC.info@ecdc.europa.eu

www.ecdc.europa.eu

Follow ECDC on social media
Twitter: @ECDC_EU
Facebook: www.facebook.com/ECDC.EU
Linkedin: www.linkedin.com/company/ecdc/

Publications Office
of the European Union