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Handbook of laboratory protocols for respiratory syncytial virus (RSV) characterisation

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Disclaimer

This handbook does not contain any assessment, recommendation or endorsement of particular commercial products; however, possible manufacturers are indicated for the reagents used in the protocols. Reagents from other manufacturers can also be used if an equivalent quality is guaranteed. If necessary, validation of the protocols must take place.

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Abbreviations

AURORAE	Laboratory Support for Influenza and Corona for Europe
ATCC	American Type Culture Collection
BSL	Biosafety level
cDNA	Complementary DNA
CPE	Cytopathic effect
dNTP	Deoxyribonucleotide triphosphate
dpi	Days post infection
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EVAg	European virus archive - GLOBAL
FBS	Fetal bovine serum
Flu	Influenza virus
g	G force
MEM	Minimum essential medium
MOI	Multiplicity of infection
NGS	Next generation sequencing
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin-Streptomycin
PFU	Plaque-forming unit
qRT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic acid
RSV	Respiratory Syncytial Virus
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
TCID50	Tissue culture infectious dose

1 Introduction

New vaccines and monoclonal antibodies against respiratory syncytial virus (RSV) represent a significant shift in the prevention of severe disease in populations at greater risk of infection, such as infants and older adults (65 years old and above). Continuous phenotypic and genomic surveillance is essential to detect mutations in pre-fusion F epitopes, monitor potential decreases in sensitivity to monoclonal antibodies and track the dynamics of RSV-A and RSV-B genotype replacement. Even for relatively stable viruses, broad immune pressure can accelerate certain evolutionary trajectories. Therefore, integrated surveillance (clinical, virological and antigenic) is critical to maintaining an optimal match between preventive strategies and circulating viruses.

This handbook has been issued by the European Centre for Disease Prevention and Control to provide laboratory professionals in the EU/EEA with a comprehensive overview of commonly used laboratory protocols for the characterisation of respiratory syncytial virus (RSV). It compiles harmonised methodologies designed to support the study of RSV biology, molecular evolution and antigenic properties across participating laboratories. The handbook serves to support the application of consistent laboratory protocols across the EU/EEA. This enables data and results generated by different laboratories to be harmonised and compared, thereby strengthening integrated RSV surveillance and the timely detection of changes in antigenic properties and susceptibility to monoclonal antibodies.

The laboratory methodologies presented in this handbook include:

- Cell culture procedures;
- Virus isolation and amplification from positive clinical specimens to generate standardised viral stocks; and
- Infectious virus quantification using RSV plaque titration assays;
- RSV-specific RT-qPCR for molecular detection and viral load measurement;
- Genome sequencing for surveillance purposes; and
- Plaque reduction neutralisation assays to evaluate resistance of viral strains to Nirsevimab, Clesrovimab or future monoclonal antibodies, or to measure anti-RSV antibody activity in human serum samples.

1.1 Disease overview

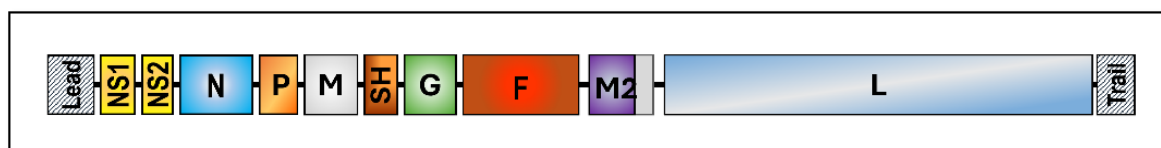
RSV is a ubiquitous and highly contagious respiratory pathogen that causes seasonal epidemics, particularly in temperate regions. Most RSV infections present with mild upper respiratory tract symptoms resembling the common cold. Populations at greater risk of infection – including infants, older adults (65 years old and above) and patients with chronic conditions (e.g. chronic obstructive pulmonary disease, heart failure or immunodeficiency) – are more likely to develop severe forms of the disease [1]. In these severe cases, RSV's pathogenicity can be the result of both viral replication that causes direct damage in the lower respiratory epithelium and from an exacerbated immune response.

Globally, RSV infections account for over three million hospitalisations and 120 000 deaths annually in children under five years old [2,3]. In premature babies and infants, the infection can lead to acute bronchiolitis or pneumonia, characterised by airway obstruction, excessive mucus production and respiratory distress; such infections may require hospitalisation or intensive care, and can even lead to death.

1.1.1 Virology

RSV belongs to the Pneumoviridae family. It is an enveloped virus with a negative-sense single-stranded RNA genome (Figure 1). The viral genome is tightly associated with the nucleoprotein (N) to form a helical nucleocapsid, which is associated with the viral replication complex consisting of the phosphoprotein (P), the large polymerase protein (L) and the M2-1 protein. The viral envelope is lined on the inner surface of the viral particle by the matrix protein (M) and contains two major transmembrane glycoproteins, the attachment protein (G) and the fusion protein (F), as well as a third transmembrane protein, the small hydrophobic protein (SH).

Figure 1. Schematic representation of the organisation of the respiratory syncytial virus genome



Genes are shown as coloured rectangles, and the names of the encoded proteins are specified. The scale is indicative. Promoter regions for replication Leader and Trailer are labeled as Lead and Trail.

It is thought that the G protein is responsible for RSV's initial attachment to respiratory epithelial cells via interactions with either the chemokine CX3C receptor 1 (CX3CR1) or heparan sulphate proteoglycans [4]. Evidence suggests that the F protein must interact with other cellular receptors (such as IGF1R and Nucleolin) for the virus to efficiently bind to the cell surface [4]. Upon virus attachment, the F protein undergoes significant conformational changes, transitioning from the pre-fusion to the post-fusion state. This process exposes the F fusion peptide, which inserts into the plasma membrane, thereby triggering fusion between the viral and cellular membranes and the release of the viral nucleocapsid into the cytoplasm. RSV infection subsequently induces the formation of inclusion bodies, also known as viral factories. These are membrane-less cytoplasmic organelles formed by the condensation of viral proteins where viral replication and transcription occur [5]. The newly synthesised viral ribonucleoproteins are then transported to the plasma membrane, where the F, G and SH proteins have been previously addressed and the budding process takes place to produce viral particles capable of initiating a new cycle of infection.

Human RSV is divided into two main antigenic subgroups, RSV-A and RSV-B. These co-circulate in human populations each year with varying degrees of dominance depending on the season and geographical region [6,7,8]. Within each of these subtypes, different lineages emerge and co-circulate for extended periods, gradually replacing each other. This process may reflect an evolutionary dynamic influenced by population immunity and selection pressure throughout epidemic seasons. The G protein exhibits high sequence heterogeneity, which can alter the virus's antigenic properties and contribute to reinfection despite previous exposure [9]. In contrast, the F protein is highly conserved, although occasional substitutions may affect the stability of its pre-fusion conformation or influence its sensitivity to neutralising antibodies.

1.1.2 Prevention

In recent years, major advances have been achieved in RSV prevention through vaccination and passive immunisation strategies targeting the F protein in its pre-fusion conformation [10].

The prevention of severe RSV infection in infants relies on passive immunisation, either through maternal vaccination or a single injection of a long-lasting monoclonal antibody. Maternal immunisation with a non-adjuvanted bivalent vaccine (Abrysvo) during pregnancy has been approved in multiple European Union/European Economic Area (EU/EA) countries to confer passive protection to infants through transplacental antibody transfer during their first months of life. Two long lasting monoclonal antibodies targeting the prefusion form of the fusion protein (Nirsevimab and Clesrovimab) provide season-long protection with a single intramuscular dose. These represent a substantial improvement over Palivizumab, an earlier monoclonal antibody that requires monthly administrations during the RSV season and is primarily reserved for infants at high risk of infection.

Three RSV vaccines have been licensed in the EU/EEA, for use in adults:

- An adjuvanted monovalent vaccine inducing cross-neutralising antibodies against the two RSV subtypes (Arexvy);
- A non-adjuvanted bivalent vaccine (Abrysvo); and
- An mRNA-based vaccine (mRESVIA).

2 Methods

The protocols presented in this guide were developed in-house at the National Reference Centre for Respiratory Viruses (Northern France) and the Molecular mechanisms of multiplication of Pneumoviruses (M3P) Unit at Institut Pasteur. The versions reproduced here correspond to the standard operating procedures currently in routine or research use in its lab.

The RSV RT-qPCR protocols have been accredited under ISO 15189 within Institut Pasteur's laboratory.

The protocol for RSV sequencing was initially developed at Institut Pasteur by Dr Etienne Simon-Loriere. The initial RSV amplicons panels are available online (https://github.com/SimonLoriereLab/RSV_amplicons_panels), and were adapted from Quick et al., 2017 [11]. The protocol was updated by the National Reference Center for Respiratory Viruses (Northern France), Institut Pasteur.

The panel presented in this handbook was optimised using RSV-positive samples circulating in Europe in 2025 and 2026. Processing samples from previous or future years – or from regions outside of Europe – may require further adjustments. Updated versions of the panel will be made available online (<https://www.pasteur.fr/fr/sante-publique/tous-cnr/virus-infections-respiratoires-dont-grippe-sars-cov-2>).

Other amplicon-based protocols are available, such as the one described in Talts et al [12].

The protocols for RSV amplification and titration have been adapted from Boullier et al [13].

3 Laboratory protocols

3.1 Biosafety

RSV is classified as a risk group 2 agent, as indicated in the EU Directive 2019/1833 amending Annexes I, III, V and VI to Directive 2000/54/EC. Consequently, it must be cultivated under Biosafety Level 2 (BSL-2) conditions, according to national regulations and regulatory authorities. Cell culture of uninfected human epithelial type 2 (HEp-2) cells also requires BSL-2 conditions, as indicated in the EU Directive 2000/54/EC [14].

3.2 General laboratory equipment and supplies

The following laboratory equipment and supplies are used in the enclosed protocols:

- PIPETBOY, pipettes, filter tips;
- 1.5 or 2 mL microcentrifuge tubes;
- 15 or 50 mL centrifuge tubes;
- Flasks and cell culture plates;
- Cryotubes;
- 96-well RT-qPCR plates (MicroAmp, EnduraPlate, Optical 96-Well Clear Reaction Plates, Applied Biosystems, A36924 or equivalent);
- 96-well PCR plates (Thermo Scientific, ref AB0900SS or equivalent);
- RNase-free water;
- Ethanol absolute;
- PBS 1X (Gibco, 149190-094);
- Trypsin-EDTA (0.05% or 0.25%) (Gibco, 25300062 or 25200056);
- MEM (1x) + GlutaMAXTM (Gibco, 41090-028);
- Fetal Bovine Serum (FBS);
- 100 U/mL Penicillin/100 µg/mL Streptomycin (Pen/Strep) (Gibco, 15140148);
- Sodium bicarbonate 7,5% (Gibco, 25080-060);
- Bleach;
- Benchtop centrifuge;
- PCR Thermocycler, such as VeritiPro Thermal Cycler, 96-well, Applied Biosystems or equivalent;
- Real-time PCR thermocycler, such as QuantStudio 5 (Thermo Fischer) or LightCycler 480 Instrument-II (Roche);
- Water bath;
- Freezing container (Corning CoolCell Freezing Container or equivalent);
- 5%-CO₂ cell incubator;
- Microscope for cell culture;
- Liquid nitrogen vapor-phase storage tanks.

Any specific equipment or reagents required are listed at the beginning of the relevant protocols.

3.3 Molecular detection and sequencing of RSV

3.3.1 RSV RT-qPCR

RT-qPCR can be performed on viral RNA extracted from clinical samples (nasopharyngeal swabs, saliva or bronchoalveolar lavage fluid), infected cell cultures or viral suspensions, for diagnostic or quantification purposes. RNA extraction from clinical samples, infected cell cultures or cell-free virus suspensions must be mixed with the lysis buffers provided by viral RNA extraction kits in the ratio defined by the manufacturers. For example, the RAV1 lysis buffer from MACHEREY-NAGEL, MagNA pure external lysis buffer from Roche, and the AVL buffer from QIAGEN. To completely inactivate the viral particles, the mixture must be incubated at 70°C for five minutes.

Materials and reagents

The materials and reagents required are:

- Total nucleic acid isolation kit (NucleoSpin 8 Virus Core Kit, Macherey-Nagel, ref 740451.4);
- Real time RT-PCR reagents (Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit, ref 11732088RFA);
- Primers and probes (Tables 2 and 4).

Method

This RNA extraction is performed manually with the NucleoSpin 8 Virus Core Kit from Macherey-Nagel. Alternative validated methods and commercial kits from other manufacturers may also be used, including those currently implemented in your facility for RNA extraction in influenza virus or SARS-CoV-2 detection. These methods may be manual or automated.

To conduct **RNA extraction** with the NucleoSpin 8 Virus Core Kit (Macherey-Nagel):

- Mix 100 µL of patient sample (e.g. fluid swabs from the anterior nose or from the nasopharynx) or viral culture with 400 µL of RAV1 lysis buffer in a biological safety cabinet. Note that 10 minutes of incubation with the lysis buffer at RT inactivates 99.99% of virus. Complete the inactivation by incubating the mixture for five minutes at 70°C to perform the rest of the procedure in a Biosafety Level 1 (BSL-1) lab.
- Add 400µL ethanol and mix.
- Load all of the mixture to the column.
- Centrifuge for two minutes at 6 000 g.
- Discard the eluate.
- Wash the column three times with the RAV3 washing buffer:
 - Wash with 500 µL of RAV3 washing buffer, centrifuge for two minutes at 6 000 g, discard the eluate.
 - Wash with 700 µL of RAV3 washing buffer, centrifuge for two minutes at 6 000 g, discard the eluate.
 - Wash with 700 µL of RAV3 washing buffer, centrifuge for 15 min at 6 000 g, discard the eluate.
- Eluate in 100µL RE elution buffer (preheated to 70°C), incubate for one minute at room temperature, centrifuge for two to three minutes at 6 000 g.

Hydrolysis probe assays for viral detection and subtyping

Two hydrolysis probe assays can be used, one for viral detection and the other for subtyping.

RSV viral detection – This multiplex RT-qPCR can detect RSV, influenza A virus and influenza B virus by targeting the M gene that is specific to each virus (panRSV-FluA-FluB triplex). The reaction mix is described in Table 1 and the primers and RT-qPCR probes in Table 2.

RSV subtyping – This RSV-A/B differential RT-qPCR can distinguish between RSV subgroups by targeting the N gene of RSV-A and RSV-B. The reaction mix is described in Table 3 and the primers and RT-qPCR probes in Table 4.

To conduct the assays, prepare the RT-qPCR reaction mixes as described in Tables 1 or 3. We recommend preparing a pre-mix with primers, probes and water. These can be aliquoted and stored at -20°C for months.

Table 1. RT-qPCR reaction mix for the detection of the M genes from RSV, influenza A virus and influenza B virus

panRSV, influenza A virus, influenza B virus triplex RT-qPCR reaction mix				
Reagents		Stock concentration	Final concentration	Volume (µL) for one well
2X reaction Buffer		–	1X	10
MgSO ₄		50 mM	0.6 mM	0.24
H ₂ O		–	–	3.64
Primer 1 Fw	VRSM-27Fw	100 µM	0.5 µM	0.1
Primer 1 Rv	VRSM-104Rv	100 µM	0.5 µM	0.1
Primer 2 Fw	GRAM-7Fw	100 µM	0.5 µM	0.1
Primer 2 Rv	GRAM-161Rv	100 µM	0.5 µM	0.1
Primer 3 Fw	GRBM-4Fw	100 µM	0.5 µM	0.1
Primer 3 Rv	GRBM-105Rv	100 µM	0.5 µM	0.1
Probe 1	VRSM-53Probe(+) FAM	100 µM	0.2 µM	0.04
Probe 2	GRAM-52Probe(+) HEX	100 µM	0.2 µM	0.04
Probe 3	GRBM-34cProbe(+) Cy5	100 µM	0.2 µM	0.04
Enzyme	Superscript III RT/Platinum Taq Mix (2U/µL)	2 U/µL	0.8 U/reaction	0.4
Sample	Purified RNA eluted in 100 µL	–	–	5
Total				20

Table 2. Primers and RT-qPCR probes for the detection of the M genes from RSV, influenza A virus and influenza B virus

Virus – gene	Name	Sequence
RSV – M	VRSM-27Fw	GGCAAATATGGAACATACGTGAA
	VRSM-104Rv	TCTTTTCTAGGACATTGTAYTGAACAG
	VRSM-53Probe(+) FAM	FAM - AGCTTCACGAAGGCTCCACATACACAG - BHQ1
Influenza A – M	GRAM-7Fw	CTTCTAACCGAGGTCGAAACGTA
	GRAM-161Rv	GGTGACAGGATTGGTCTTGTCTTTA
	GRAM-52Probe(+) HEX	HEX - TCAGGCCCCCTCAAAGCCGAG - BHQ1
Influenza B – M	GRBM-4Fw	TCGCTGTTTGGAGACACAATTG
	GRBM-105Rv	CAAGGCAGAGTCTAGGTCAAATTCT
	GRBM-34cProbe(+) Cy5	Cy5 - AGAAGGCAAAGCAGAACTAGC - BHQ3

RSV M primers originate from Wang et al [15], and the RSV M probe was designed in-house at the National Reference Center for Respiratory Viruses (Northern France), Institut Pasteur. Influenza A M primers and probe originate from Terrier et al [16] and the [WHO information for molecular detection of influenza virus](#) [17]. Influenza B M primers originate from Jørgensen et al [18] and the Influenza B probe was designed in-house at the National Reference Center for Respiratory Viruses (Northern France), Institut Pasteur.

Table 3. RT-qPCR reaction mix for RSV-A/B differential RT-qPCR

RSV-A/B differential RT-qPCR mix				
Reagents		Stock concentration	Final concentration	Volume (µL)
2X reaction Buffer		–	1X	10
MgSO ₄		50 mM	0.6 mM	0.24
H ₂ O		–	–	4.08
Primer 1 Fw	VRSN-1141Fw	100 µM	0.5 µM	0.1
Primer 1 Rv	VRSN-1262Rv	100 µM	0.5 µM	0.1
Probe 1	VRSAN-1262Probe(+) FAM	100 µM	0.2 µM	0.04
Probe 2	VRSBN-1262Probe(+) Atto647N	100 µM	0.2 µM	0.04
Enzyme	Superscript III RT/Platinum Taq Mix (2U/µL)	2 U/µL	0.8 U/reaction	0.4
Sample	Purified RNA eluted in 100 µL	–	–	5
Total				20

Table 4. Primers and RT-qPCR probes for the differential detection of the N genes from RSV-A and RSV-B

Virus – gene	Name	Sequence
RSV – N	VRSN-1141Fw	ATGGCTCTTAGCAAAGTCAAGT
	VRSN-1262Rv	TGCACATCATAATTRGGAGTRTCA
	VRSAN-1262Probe(+) FAM	FAM – ACACCTCAACAAAGATCAACTTCTRTCATCCAGCA – BHQ1
	VRSBN-1262Probe(+) Atto647N	ATTO647N – ACATTAAATAAGGATCAGCTGCTGTCATCCAGCA – BHQ3

Primers and probes adapted from Wang et al [11].

RT-qPCR programme

The RT-qPCR is performed in a thermocycler with the thermocycling programme described in Table 5.

Table 5. Thermocycling programme for RT-qPCR

Step	Temperature	Duration	Number of cycles
Reverse Transcription	50°C	15 minutes	1
Denaturation	95°C	2 minutes	1
Elongation ^a	95°C	15 seconds	50
	60°C	40 seconds	
Cooling	40°C	30 seconds	1

^a Measure the fluorescent signal at the end of this step.

To analyse the data, set the threshold line manually, depending on your specific thermocycler. Always use the same threshold once it is determined for your instrument. Locate the Ct on the amplification curve (i.e. the cycle number where the curve crosses a pre-defined threshold).

3.3.2 RSV sequencing

The reference sequence for RSV-A is the A/England/397/2017 strain, which is available in GenBank and GISAID under the accession numbers PP109421 and EPI_ISL_412866, respectively.

The reference sequence for RSV-B is the B/Australia/VIC-RCH056/2019 strain, which is available in GenBank and GISAID under the accession numbers [OP975389](#) and EPI_ISL_1653999, respectively.

Materials and reagents

The materials and reagents required are:

- dNTPs 10mM (Thermo Scientific, ref R0192);
- RNase inhibitor (Promega, N2511);
- Reagents for cDNA synthesis: LunaScript RT Supermix Kit, NEB, ref E3010;
- Reagents for PCR amplification: Q5 High-Fidelity DNA Polymerase kit, NEB, ref M0491;
- Primers (Tables 5 and 6);
- AMPure XP beads (Beckman Coulter, ref A63880);
- Magnetic rack.

Method

Whether or not the RSV sequencing will be successful relies on the viral load. In our experience, samples with a Ct inferior to 28 in RSV-A/B differential RT-qPCR are successfully sequenced. This threshold might depend on local settings and has to be adapted to local sequencing strategy.

cDNA synthesis

To perform the cDNA synthesis:

- Mix the reagents given in Table 6.
- Add a sample with water as a negative control for the whole procedure.
- Perform the reverse transcription with the thermocycling programme in Table 7.

Table 6. Reagents for cDNA synthesis

Reagent	1X
Lunascript Supermix 5X (NEB, ref E3010)	2 µL
RNA ^a	8 µL
Total	10 µL

^a Extract RNA as described above, from 100 µL of respiratory sample and eluted in 100 µL.

Table 7. Thermocycling programme for cDNA synthesis

Temperature	Time
25°C	2 minutes
55°C	10 minutes
95°C	1 minute
4°C	∞

PCR amplification

For the PCR amplification, generate the amplicons as independent duplicates. Pool primers beforehand as described in Tables 10 and 11.

Mix the reagents given in Table 8 and perform the PCR multiplex reaction with the thermocycling programme in Table 9.

Assess the DNA amplification on agarose gel: run 5 µL of each reaction on a 1% agarose gel. The PCR products should produce a visible ~400 bp band and the negative control (no cDNA) should be clean.

Pool the multiplex PCR products 1 and 2 (50µL final volume).

Table 8. Reagents for PCR amplification

Composition	Reaction	Final concentration in 25 µL
5X Q5 reaction buffer (NEB)	5 µL	1X
10mM dNTP	0.5 µL	200 µM
50X primer pool 1 or primer pool 2	0.5 µL	–
cDNA	2.5 µL	–
Q5 polymerase (2000 U/mL) (NEB)	0.25 µL	0.02 U/µL
H2O RNase DNase free	For final volume 25 µL	–

Table 9. Thermocycling programme for PCR amplification

T°	Time	Number of cycles
98°C	30 s	1
98°C	15 s	35
65°C	5 min	
10°C	∞	1

Purification of amplicons on beads

To perform purification of amplicons on beads:

- Allow the AMPure XP beads to warm up to room temperature for 30 minutes.
- Vortex the beads for 10 seconds.
- Add 72 µL (1,8X) of beads to the 50µL sample.
- Mix thoroughly by vortexing for 10 seconds.
- Incubate samples 10 minutes at room temperature to allow the samples to bind to the beads.
- Place the tubes on an appropriate magnetic rack to separate beads from the supernatant.
- When the solution is clear (about 5–10 minutes), discard the supernatant without disturbing the beads.
- Wash: keep the samples on the magnet and add 200 µL of freshly prepared 70% ethanol.
- Incubate at room temperature for one minute.
- Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads.
- Repeat once for a total of two washes.
- Completely remove the residual ethanol.
- Allow the samples to dry at room temperature for 5–10 minutes, until the beads are dry.
- Add 40 µL PCR-grade water on the beads.
- Remove the samples from the magnet.
- Vortex the beads for 10 seconds.
- Place the samples on a magnetic particle collector to collect the beads. Incubate until the solution is clear.
- Carefully transfer the 40 µL supernatant to a clean tube.
- Quantify the DNA concentration using the Qubit High Sensitivity DNA kit (or equivalent) from 1 µL of each product. Expected range is 10–100 ng/µL of DNA. Samples can be stored at -20°C.

Alternative techniques can also be used to purify amplicons.

Amplicon sequencing

For the amplicon sequencing, prepare two 50X concentrated pre-mixes: primer pool 1 and primer pool 2. Aliquot and freeze at -20°C until use. Freeze/thaw the aliquots a maximum of 5 times to prepare PCR mixes.

Table 10. Primers for RSV-A tiled amplicon sequencing – version 20 March 2026

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (mM) (50 X)
RSVA_1_LEFT	CTTGCGTAAACCAAAAAATGGGG	1	30	1.50
RSVA_1_RIGHT	TGGTCATTGTTGAATCACTTAGTTTTTG	1	30	1.50
RSVA_3_LEFT	TAGAGACATCATAACACATAAATTTATATACTTG	1	15	0.75
RSVA_3_RIGHT	CAACTTGACTTTGCTAAGAGCCAT	1	15	0.75
RSVA_5_LEFT	AGGTATGTTATATGCTATGTCTAGATTAGGA	1	75	3.76
RSVA_5_RIGHT	TGGGTAATAAACCTTTATAGCGTTTCA	1	75	3.76

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (mM) (50 X)
RSVA_7_LEFT	AAGTGATGTTACGGTGGGGG	1	15	0.75
RSVA_7_RIGHT	ACTCAAAGCTCTACATCATTATCTTTTGG	1	15	0.75
RSVA_9_LEFT	AAACCCAATAAATGAGACAGATGATACT	1	15	0.75
RSVA_9_RIGHT	TCCTGAGTCTTGCCATAGCTTC	1	15	0.75
RSVA_11_LEFT	GCTACCAAACGTACATCAAAACACA	1	8	0.38
RSVA_11_LEFT_bis	GCTACCAAACGTATATCAAAACACA	1	8	0.38
RSVA_11_RIGHT	TCTTAATGAGGGTCCCTTGGGT	1	15	0.75
RSVA_13_LEFT	ACACTCAACCCAACACATGACA	1	113	5.64
RSVA_13_LEFT_bis	ACACTCAACCCACACATGACA	1	38	1.88
RSVA_13_RIGHT	TTGTATGAATCTACTCATTGATGTAGAGG	1	113	5.64
RSVA_13_RIGHT_bis	TTGTGTGAATCTGCTCATTGATGTAGAGG	1	38	1.88
RSVA_15_LEFT	ACACGCTAGATAAAATCAACCAATGG	1	15	0.75
RSVA_15_RIGHT	CATTTGCCCCAGCGTTRTTYTT	1	15	0.75
RSVA_17_LEFT	CCACAAAGTCACACTAACAACCTGC	1	15	0.75
RSVA_17_RIGHT	TGGTGGTGGTTTTCTTTCCAGG	1	15	0.75
RSVA_19_LEFT	AAACCCTCCACTCAACCACCTC	1	15	0.75
RSVA_19_RIGHT	TCTGTACCATTACACTTATTTTCCTTGAT	1	15	0.75
RSVA_21_LEFT	GGCATTGCCGTATCCAAGGT	1	15	0.75
RSVA_21_RIGHT	TGCTGTCTAACTATTTGAACATTGCT	1	15	0.75
RSVA_23_LEFT	GTGACAATGCAGGATCAGTATC	1	15	0.75
RSVA_23_RIGHT	CAGAGGGGAACACTAATGGATC	1	15	0.75
RSVA_25_LEFT	TATACTGCAAGGCCAGAAGCAC	1	15	0.75
RSVA_25_RIGHT	CATGGGGTGGCCATTCAAAATAA	1	15	0.75
RSVA_27_LEFT	ACTGAACTCAACAGCGATGACA	1	75	3.76
RSVA_27_RIGHT	CATTGAATGGTTGATCCGGTGG	1	75	3.76
RSVAb_29_LEFT	CTCAGAATGTAACGCTTTAGGAAG	1	150	7.52
RSVAb_29_RIGHT	GTAGTAATGACTGAGTTGTCTTCATC	1	150	7.52
RSVAb_31_LEFT	GCAACATCCTCCATCATGGTTAATAC	1	75	3.76
RSVAb_31_RIGHT	GTAGAACCCCTCATTGTGGAATAG	1	75	3.76
RSVA_33_LEFT	TGCAGGTGACAATAACCTCAACA	1	30	1.50
RSVA_33_LEFT_bis	TGCAGGTGACAACAACCTCAACA	1	30	1.50
RSVA_33_RIGHT	AAATTTTtaggaggtgatatagccttatca	1	30	1.50
RSVA_33_RIGHT_bis	AAATTTTtaggaggtgatatagccttgatca	1	30	1.50
RSVA_35_LEFT	AGGCAAAGAAAGAGAACTCAGTGT	1	15	0.75
RSVA_35_RIGHT	GGGGGTGCATGCCTATATGT	1	15	0.75
RSVA_37_LEFT	TCACTATTAGATCTAATATCTCTCAAAGGGA	1	150	7.52
RSVA_37_RIGHT	ACTGCATAATAGACTTTCACCTCTATATTC	1	150	7.52
RSVA_39_LEFT	TCGAAGTTTCTATAGAAGAACTCCTGA	1	15	0.75
RSVA_39_RIGHT	TCATAAACAACCTTAGCCCCGTGA	1	15	0.75
RSVA_41_LEFT	GAGATAAAAGAGAAATATTGAGTATGGAAAACC	1	75	3.76
RSVA_41_RIGHT	TGGCCTTCTCATATGTTAACCCA	1	75	3.76
RSVA_43_LEFT	TGGCCTTAGCTTAATGTCTGTAGT	1	15	0.75
RSVA_43_RIGHT	ACATATGATCAGTTATATATCCCTCTCCC	1	15	0.75
RSVA_45_LEFT	AGCTTCAAACATATGGTTTCTTAAACG	1	150	7.52

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (mM) (50 X)
RSVA_45_RIGHT	CCTATACAATAGTCACTCAGTGTC	1	150	7.52
RSVA_47_LEFT	CCTTAGTGCACAATAGCACATCAC	1	15	0.75
RSVA_47_RIGHT	CAGGCAATTCAGCATCACAGAC	1	15	0.75
RSVA_49_LEFT	GGTCCTGCAAATGTGTTCCCA	1	75	3.76
RSVA_49_RIGHT	AGCTGTTTAAACAATTCACCTTAGATGAGG	1	75	3.76
RSVA_2_LEFT	TGGCCTAATAGATGACAATTGTGAAA	2	30	1.50
RSVA_2_RIGHT	TGACCAGAAATGTAAATGTGGCCT	2	30	1.50
RSVA_4_LEFT	CAAACAATCCGAAATAACAACCTTTATGCA	2	15	0.75
RSVA_4_LEFT_bis	CAAACAATCCAAAATAACAGCTTTATGCA	2	15	0.75
RSVA_4_RIGHT	TCCACTCCATTTGCCCTAACATGA	2	15	0.75
RSVA_4_RIGHT_bis	TCCACTCCATTTGCTTTGACATGA	2	15	0.75
RSVA_6_LEFT	AGCAGCAGGAGATAGATCAGGT	2	15	0.75
RSVA_6_RIGHT	GGTTGTTCAATATATGGTAGAATCCTGC	2	15	0.75
RSVA_8_LEFT	AAATGGTGTGATTAACCTACAGTGATTAGA	2	11	0.56
RSVA_8_RIGHT	GGCGTAGGGTCTTCTTTGAACT	2	11	0.56
RSVA_10_LEFT	AGGATTGATGAGAAATTAAGTGAAATACTAGG	2	15	0.75
RSVA_10_RIGHT	TCAGCAGATAGATGTTTGTTGGA	2	15	0.75
RSVA_12_LEFT	TCCATGCCAGCAGATCTACTCA	2	15	0.75
RSVA_12_RIGHT	TCAAGTGTGTTCAGATCTTTATTCTGA	2	15	0.75
RSVA_14_LEFT	CAACAAATTGGAAGCACACAGCT	2	15	0.75
RSVA_14_RIGHT	TGTTATCATGTGTATTAGTGTAAGTAAGGC	2	15	0.75
RSVA_16_LEFT	TCTCACCATGCAAGCCATYATC	2	15	0.75
RSVA_16_RIGHT	TGGGTGAGGTATGTTGGGGT	2	15	0.75
RSVA_18_LEFT	ACCCAACAATGATTTTCACTTTGAAGT	2	15	0.75
RSVA_18_RIGHT	GTGTTGGATGAAGATRGAGRTTGTGAT	2	15	0.75
RSVA_20_LEFT	GCAAAGGCTATCTTAGTGCTCTAAG	2	15	0.75
RSVA_20_RIGHT	AGCCTTGTGTTGTGATAGTAGAGC	2	15	0.75
RSVAb_22_LEFT	GTGTAACACACCTGTAAGCAC	2	75	3.76
RSVAb_22_RIGHT	CAGAGATTTACCTCACTTGGAATG	2	75	3.76
RSVA_24_LEFT	GGGTGGATACTGTGTCTGTAGG	2	15	0.75
RSVA_24_RIGHT	GGTGCTATTTTTATTAGTTACTAAATGCA	2	15	0.75
RSVA_26_LEFT	GGGGCAAATATGTCGAAGGA	2	15	0.75
RSVA_26_RIGHT	GCTTTCAATATATGATATGACAGTATTGTACAC	2	15	0.75
RSVA_28_LEFT	CAAGTAATTGTAGAGTCACTATGTATAACCA	2	15	0.75
RSVA_28_LEFT_bis	CAAGTAATTGTAGAGTCACTATGTATAATCA	2	15	0.75
RSVA_28_RIGHT	AGTTGGTATAATCATTTTTGAGATAAGGACC	2	30	1.50
RSVAb_30_LEFT	CTTATGACATACAAGAGTATGACCTC	2	15	0.75
RSVAb_30_RIGHT	CTCACTAGATCGATATTGTGTTAATATGC	2	15	0.75
RSVA_32_LEFT	TGTGGATTCAATAATGTTATCTTGACACA	2	15	0.75
RSVA_32_RIGHT	AGCATCCATGGCTTGCTTTCA	2	15	0.75
RSVA_34_LEFT	TCAGGACTACGTTTCTATCGAGAGT	2	15	0.75
RSVA_34_RIGHT	TGTTTTCTGCTATCATTTTCTCTGCT	2	15	0.75
RSVA_36_LEFT	TGGATGAAGTGCATGGGTGTACA	2	30	1.50
RSVA_36_RIGHT	TGTGGCCTATTCTGCATACTC	2	30	1.50

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (mM) (50 X)
RSVA_38_LEFT	GGACCGTGGATAAACTACTTGA	2	56	2.82
RSVA_38_LEFT_bis	GGACCGTGGATAAACACCATACTTGA	2	19	0.94
RSVA_38_RIGHT	CTTGAAGTTTGTCTTTAAATCATGGTTTG	2	75	3.76
RSVA_40_LEFT	GTGCACAACACTATACCACCAC	2	15	0.75
RSVA_40_RIGHT	TGTCCATTGTATACATGATACTGGGT	2	15	0.75
RSVA_42_LEFT	AGCAAAATTGGATTGGGTGTATGC	2	15	0.75
RSVA_42_RIGHT	ACATCACCTGTGAATATGGGAGG	2	15	0.75
RSVA_44_LEFT	ACTAATTTAGCTGGACATTGGATTCTT	2	30	1.50
RSVA_44_RIGHT	TGTTAACAACCCAAGGGCAAAC	2	30	1.50
RSVA_46_LEFT	ACAACAAATTATATCATCCYACACCAG	2	150	7.52
RSVA_46_RIGHT	TATGATGCCAAGGAAGCATGC	2	150	7.52
RSVA_48_LEFT	GACCATTCTGCTACAGATGCA	2	30	1.50
RSVA_48_RIGHT	GGCATGATGAAATTTTGGTTCTTGA	2	30	1.50
RSVA_50_LEFT	CGATAAAGAGTCTATTGATGCAAATTAAGAG	2	75	3.76
RSVA_50_RIGHT	TGGATTTAACTTATTCTCCTAGATCAAAA	2	75	3.76

Table 11. Primers for RSV B tiled amplicon sequencing – version 26 May 2025

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (50X) (mM)
RSVB_1_LEFT	CAGAAATGGGTGCAATCACTG	1	15	0.75
RSVB_1_RIGHT	TCATGAATGGAGATCAAGCCCAA	1	15	0.75
RSVB_3_LEFT	ACCATGAGCACTACAAAMGACAA	1	30	1.50
RSVB_3_RIGHT	GTTGGAAGTTACGGGTGAGGT	1	30	1.50
RSVB_5_LEFT	TGACACTCCAATTATGATGTGCA	1	30	1.50
RSVB_5_RIGHT	TTACAAGGCAGCTATACACAGT	1	30	1.50
RSVB_7_LEFT	AGCACAATCATCCACAAGAGGG	1	224	11.22
RSVB_7_RIGHT	GAGTTGCTCTGCATATGCTTTGG	1	224	11.22
RSVB_9_LEFT	TCCAAAGATCCTAAGAAGAAAGATAGCA	1	11	0.56
RSVB_9_RIGHT	TATTCCGTCACGAGCCGAAGTR	1	11	0.56
RSVB_11_LEFT	TTGGAAGACAACGATAGCGACAA	1	15	0.75
RSVB_11_RIGHT	GCTTTACTAGTATGTTGATGCTTGCA	1	15	0.75
RSVB_13_LEFT	TGCAGTTTAACATGCTTAAAGTAAAAAG	1	30	1.50
RSVB_13_RIGHT	GTGGTTTGATTGAAAACGTGTAGC	1	30	1.50
RSVB_15_LEFT	CCTAGAGTGCGAATAGGTAAATAAAACAAG	1	150	7.48
RSVB_15_RIGHT	TGTGATGCCATGACTCTGTGAG	1	150	7.48
RSVB_17_LEFT	GAAAAGACCTGGGATACTCTTAATCATCT	1	75	3.74
RSVB_17_RIGHT	GTGCTTGGCTTGTGTCTGTG	1	75	3.74
RSVB_19_LEFT	AGAAACCACCTTAACCAACAAAAA	1	15	0.75
RSVB_19_RIGHT	GCAAGAGTTAGGAAGATTGCACTTG	1	15	0.75
RSVB_21_LEFT	CAGAGTTACTTGAGTGCTTTAAGAAC	1	150	7.48
RSVB_21_RIGHT	CTTTGTTTCGTAAGCTGCAAAGCA	1	150	7.48
RSVB_23_LEFT	AGCACTTACATGTTAACAACAGTGAG	1	30	1.50

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (50X) (mM)
RSVB_23_RIGHT	ACAAAGGCTGACTTCACTTGGT	1	30	1.50
RSVB_25_LEFT	GCTGTGATTATGTGTCAAACAAGGA	1	30	1.50
RSVB_25_RIGHT	TGCAATATTATTGATTCCACTTAGTTGGTC	1	30	1.50
RSVB_27_LEFT	TACATCAACATCACADCACAGGC	1	150	7.48
RSVB_27_RIGHT	CTCTCTAGCACTCCAACATATACCAAG	1	150	7.48
RSVB_29_LEFT	CTTAAACGATTACCAGCAGACGTR	1	15	0.75
RSVB_29_RIGHT	GGTTGGATGATATAGAATCGTTATGATCAAG	1	15	0.75
RSVB_31_LEFT	ACGGCCCTTATCTTAAAAATGATTATACC	1	15	0.75
RSVB_31_RIGHT	ACCAGTGATTAACCATGATGGAGG	1	15	0.75
RSVB_33_LEFT	AGATAATCAAACTTTGAGTGGTTTTCACT	1	15	0.75
RSVB_33_RIGHT	ACTCTTGATAGTAGATCCTTTTGAGCC	1	15	0.75
RSVB_35_LEFT	ACTTGAGTGAGCTATATTTCTCTTCAGA	1	15	0.75
RSVB_35_RIGHT	ACTAGTCCATATTAGATCTTTTGAGGAG	1	15	0.75
RSVB_37_LEFT	AGAAAGAGAGCTCAGTGTGGGT	1	15	0.75
RSVB_37_RIGHT	TCCTTTATAAAAGGAGGTGCATGTCT	1	15	0.75
RSVB_39_LEFT	CACAGCTCTGATAAATGGTGATAATCAG	1	15	0.75
RSVB_39_RIGHT	TTGCAGAGCAATTTGATTGTATAACCA	1	15	0.75
RSVB_41_LEFT	GAGAACTCCAGACTTCCTTACAGAR	1	75	3.74
RSVB_41_RIGHT	GCGCACTTTTAGAAAATATTTTGTTGGA	1	75	3.74
RSVB_43_LEFT	TGTAAGAGAAAGATCTTGGTCATTATCCA	1	15	0.75
RSVB_43_RIGHT	ACTGTTAACCGGTGTAATAATTKACAC	1	15	0.75
RSVB_45_LEFT	CTTAGCTTGATGTCAGTWTGGAAC	1	15	0.75
RSVB_45_RIGHT	TGATCAGTTATATACCCYCTCCCC	1	15	0.75
RSVB_47_LEFT	GCTACTGGAATCTATGTCTAAAGTTTTCC	1	37	1.87
RSVB_47_RIGHT	GGGGTTGGGTGATAGTTTGTTRTAATT	1	37	1.87
RSVB_49_LEFT	GTGATAGACAGGATTATAGATCATTCAGGT	1	15	0.75
RSVB_49_RIGHT	GTTGCATCTGTAGCAGGAATGGT	1	15	0.75
RSVB_51_LEFT	ACAGCCAATTGGAGTAAATYATAATTGA	1	15	0.75
RSVB_51_RIGHT	TCTCCATTAACACTCTTCAATTTTGAC	1	15	0.75
RSVB_53_LEFT	AACAAGCTTATAAACCACAAGCATATGA	1	150	7.48
RSVB_53_RIGHT	TGTCTCGTTGTGTTGTAATGCAC	1	150	7.48
RSVB_2_LEFT	AGTGAAATCTAACTTACAACAATGCCA	2	72	3.61
RSVB_2_RIGHT	GGTGAGAGATGTTATTATTGAATCCATCG	2	72	3.61
RSVB_4_LEFT	TGGCGGGTTCTAGAATGTATTGG	2	29	1.44
RSVB_4_RIGHT	TGATTTGCATCTTCAGTGATTAATAGCA	2	29	1.44
RSVB_6_LEFT	AAGCTTGACATCAGAAATACAAGTCAA	2	14	0.72
RSVB_6_RIGHT	CTTGTCTGTAACCATAGGCATTCA	2	14	0.72
RSVB_8_LEFT	AGCAGGTCTAGGCATAATGGGA	2	14	0.72
RSVB_8_RIGHT	TGTTGGTGCCAGATGTTATCGG	2	14	0.72
RSVB_10_LEFT	TGACCAACAAATGATAACATTACAGCA	2	14	0.72
RSVB_10_RIGHT	GGTGTCTGTTTTGTTGATGYTGT	2	14	0.72
RSVB_12_LEFT	CAGCAGCTGTCCAGTACAATGT	2	14	0.72
RSVB_12_RIGHT	TTCACATAGAGCAATGATCTCATGAGT	2	14	0.72

Primer	Sequence	Pool	Final concentration (nM)	Concentration pre-mix (50X) (mM)
RSVB_14_LEFT	GGAGCATTCAAGTATATCAAGCCAC	2	29	1.44
RSVB_14_RIGHT	GCTTGTGAATTCTATTGTRATGGATGT	2	29	1.44
RSVB_16_LEFT	ACAGATGTATCAAAATCGACACATAGTG	2	14	0.72
RSVB_16_RIGHT	GCAGAGATGATGAATATTATGGCTGC	2	14	0.72
RSVB_18_LEFT	ACCCAATACAAAATCAGAAACACACC	2	14	0.72
RSVB_18_RIGHT	ACAGTGGATTGTGGGGTGCT	2	14	0.72
RSVB_20_LEFT	GTGATCCCTCAAGCAAGAACKA	2	72	3.61
RSVB_20_RIGHT	TGATTGTATAGTTCATATACTGTGGTGCT	2	72	3.61
RSVB_22_LEFT	AGCAAGTGGTATAGCTGTATCCAAAG	2	144	7.22
RSVB_22_RIGHT	GCCTTACTATCTGAACATTGCTTGAC	2	144	7.22
RSVB_24_LEFT	TCCTTCTTTCCACAAGCTGACAC	2	14	0.72
RSVB_24_RIGHT	GAAGGAAACACTAGAGGGTCATAGT	2	14	0.72
RSVB_26_LEFT	CAAAGTTTAGCTTTTATTCGTAGATCYGA	2	14	0.72
RSVB_26_RIGHT	GTGTTGATTGCGATATTTGAGGCTT	2	14	0.72
RSVB_28_LEFT	TGTTAAACAAGATACTCAAGTCAATGGAC	2	14	0.72
RSVB_28_RIGHT	TGGTTGACTCTTTGGGTTGCT	2	14	0.72
RSVB_30_LEFT	GCCACTCAACAATTTCTCCAACA	2	14	0.72
RSVB_30_RIGHT	AGTAATGACTGGAAATAAGTTGGTCTTC	2	14	0.72
RSVB_32_LEFT	GTGATGAAACTCAGTACTTACAACCA	2	29	1.44
RSVB_32_RIGHT	GCTGATGTCTTTCCATGTCAAAATTG	2	29	1.44
RSVB_34_LEFT	CTATCACAAATTATCCTTACGGAGATTGT	2	14	0.72
RSVB_34_RIGHT	TCATTGCAGTTAATTCTTACAGCATCC	2	14	0.72
RSVB_36_LEFT	TTGCCGTTCTATCGTGAGTTTCA	2	29	1.44
RSVB_36_RIGHT	CAGGGAAGAATTGTAAATATTTTCGGC	2	22	1.08
RSVB_36_RIGHT_bis	CAGGGAAGAATTGTAAATATTTTCAGC	2	7	0.36
RSVB_38_LEFT	ACGAACTGCATGGRGTACAATC	2	14	0.72
RSVB_38_RIGHT	TCCCTTAAGCTTATGGCCTATGC	2	14	0.72
RSVB_40_LEFT	AGGTAGCTTAACACAGGARTTAGAATAC	2	108	5.42
RSVB_40_RIGHT	GACACAAGTCAAGAATTTGTTCACTCT	2	108	5.42
RSVB_42_LEFT	ATCCACAGGCTTTAGGGTCTGA	2	14	0.72
RSVB_42_RIGHT	GGCTATAGTGCTAGTTGTATATTTAATRTCC	2	14	0.72
RSVB_44_LEFT	CAAAGATGAATTCATGGAAGAACTGAGT	2	14	0.72
RSVB_44_RIGHT	GCTTCAACTTGATGATATCRACATCTCC	2	14	0.72
RSVB_46_alt_LEFT	TTAGCTGGACATTGGATTCTGA	2	72	3.61
RSVB_46_RIGHT	CAACCCAAGGGCATACGGT	2	54	2.71
RSVB_46_RIGHT_bis	CAACCCAAGGGCATACAGT	2	18	0.90
RSVB_48_LEFT	CTTTTCAGACAACACTCATCTGCT	2	72	3.61
RSVB_48_RIGHT	CTGATCTTGCATCCTGTGGAAC	2	72	3.61
RSVB_50_LEFT	CCCAGTTGTATAGCATTCATAGGTGA	2	29	1.44
RSVB_50_RIGHT	TTTGAAATCAATATCATCTTGAGCATGGT	2	29	1.44
RSVB_52_LEFT	GATGTTGTGCAAAATGCTAAATTGATTCT	2	14	0.72
RSVB_52_RIGHT	CACTACCTGTTATTTAATCAGCTTCTTGA	2	14	0.72

Library preparation and sequencing

The amplicon panel is compatible with Oxford Nanopore Technologies and Illumina sequencing. Illumina DNA sequencing can be performed using the Nextera kit for library preparation and the NextSeq 2000 sequencing system (Illumina Inc.).

If using a combined paired read length shorter than the amplicon's size (~400nt) with DNA fragmentation or tagmentation, adapt the bioinformatic pipeline for primer trimming. It is recommended that at least 150 nt long reads are used.

3.3.3 Cell culture

HEp-2 cells are the reference cell line for the isolation and amplification of RSV. All cell cultures are performed in a biosafety cabinet using sterile materials to prevent bacterial contamination.

Cells should be routinely tested for mycoplasma, as described below.

Materials and reagents

The materials and reagents required are as follows.

Optional for in-house mycoplasma testing:

- VenorGem OneStep kit from Minerva Biolabs (Cat No:118025).

HEp-2 cells:

- CCL-23¹.

Complete growth medium:

- MEM (1x) + GlutaMAX (Gibco, 41090-028):
 - 10% FBS;
 - 100 U/mL Penicillin / 100 µg/mL Streptomycin (Pen/Strep) (Gibco, 15140148).

The composition of the media may vary depending on the laboratory. Ideally, once a cell culture medium has been chosen, it should not be changed.

Freezing medium:

- 80% FBS;
- 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich, D2650);

The FBS must be heat-inactivated for 30 minutes at 56°C before use.

Method

HEp-2 cell passaging

A sub-cultivation ratio of 1:5 to 1:10 twice a week is recommended for HEp-2 cells. Adjust the seeding density based on the observed growth kinetics in your lab to ensure cultures reach ~90% confluence on the scheduled passage day.

To conduct cell passaging:

- Assess the cell confluence under a microscope.
- Aspirate culture media and rinse once with PBS 1X.
- Add 0.05% trypsin-EDTA to just cover the cells (around 3 to 5 mL for a T-150 flask, for example) and incubate for 5–10 minutes at 37°C.
- Collect the cells and resuspend them in complete growth medium.
- Seed cells (1/5 to 1/10) in a new flask with fresh growth culture medium.
- Maintain cells at 37°C in a 5% CO₂ incubator.

HEp-2 cell seeding

To plate cells for infection and titration, proceed as described for cell passaging. After resuspending the cells in growth media, count the cells and plate them in complete growth medium according to Table 12. Cells should reach 90% confluence the next day.

¹ American tissue culture collection (ATCC) CCL-23. Available at: <https://www.atcc.org/products/ccl-23>.

Table 12. HEp-2 cell seeding and infection conditions

Flask, dish or plate	Surface	Nb HEp-2 plated the day before	Volume medium	Volume inoculum for adsorption
T-150	150 cm ²	15 x 10 ⁶	30mL	5 mL
T-100	100 cm ²	10 x 10 ⁶	20mL	3 mL
T-75	75 cm ²	7.5 x 10 ⁶	15 mL	2.5 mL
100 mm dish	78 cm ²	5 x 10 ⁶	10 mL	2 mL
T-25	25 cm ²	2.5 x 10 ⁶	5 mL	1 mL
35 mm dish	8.8 cm ²	1 x 10 ⁶	4 mL	400 µL
6-well	9.6 cm ²	1 x 10 ⁶	2.0 mL	400 µL
12-well	3.5 cm ²	5 x 10 ⁵	1 mL	400 µL
24-well	1.9 cm ²	2.5 x 10 ⁵	0.5 mL	200 µL
48-well	1.1 cm ²	1 x 10 ⁵	0.2 mL	100 µL
96-well	0.32 cm ²	0.5 x 10 ⁵	0.1 mL	100 µL

Mycoplasma testing

Various assays – such as fluorescent staining, ELISA, PCR, immunostaining or microbiological assays – are available for detecting mycoplasma in cell cultures. The two methods detailed below are provided as examples; any validated alternative that is routinely used in your facility is also acceptable.

Commercial testing

Mycoplasma testing services are available from multiple companies, such as Eurofins Scientific.

To prepare the samples:

- Collect 500 µL of cell culture supernatant.
- Incubate for 10 minutes at 90°C.
- Centrifuge at 10 000 g minimum for 10 seconds.
- Transfer 100 µL of the supernatant to a new reaction vessel.

Barcode the samples and send them to mycoplasma testing companies, where DNA extraction and PCR-based detection are performed.

PCR mycoplasma testing

The VenorGem OneStep kit from Minerva Biolabs (Cat. No.: 11-8025) can be used for in-house mycoplasma testing.

To prepare the samples:

- Inactivate 100µL of cell culture supernatant for 10 minutes at 95°C.
- Prepare the VenorGem OneStep Mix according to the manufacturer's instructions.
- Add 2 µL of inactivated sample to 23 µL of VenorGem OneStep Mix.
- Include positive and negative controls in the whole process.
- Perform the thermal amplification cycle in a thermocycler with the thermocycling programme given in Table 13.

Analyse PCR products with a 1% agarose gel electrophoresis.

Samples are considered positive (strong contamination) when a clear band is detected at the same height as the positive control (expected size: 265–278 bp).

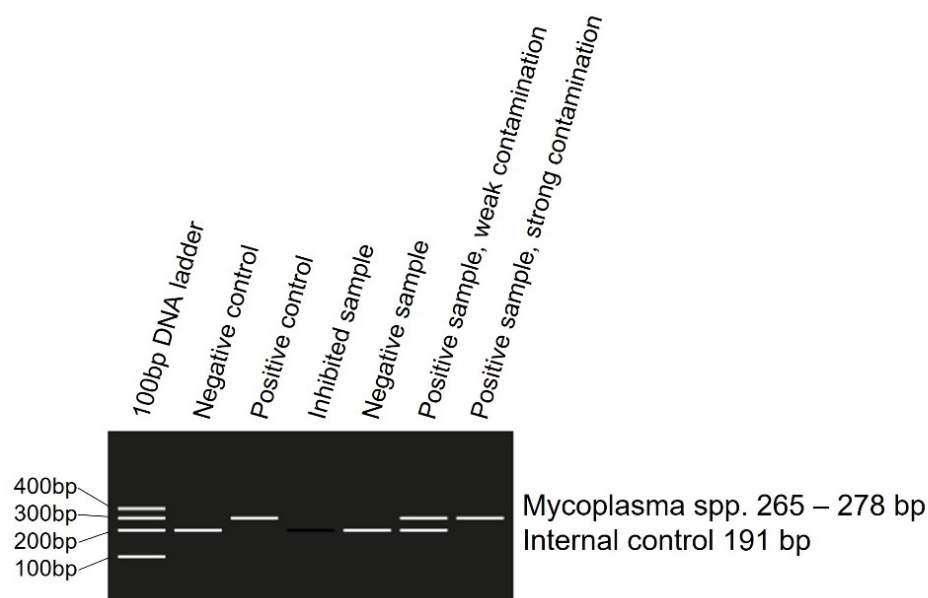
Samples are considered positive (weak contamination) when two bands are detected, one at the same height as the positive control and one at the same height as the negative control (expected sizes: 265–278 bp and 191 bp, respectively).

Samples are considered negative when a band is detected at the same height as the negative control (expected size: 191 bp).

A visualisation of possible sample results is given in Figure 2.

Table 13. Thermocycling programme for PCR mycoplasma testing

Temperature	Time	Number of cycles
94°C	2 minutes	1
94°C	30 seconds	39
55°C	30 seconds	
72 °C	30 seconds	
10°C	∞	1

Figure 2. Visualisation of possible sample results

Source: Manufacturer's instruction manual (available online at: <https://minerva-biolabs.com/en/mycoplasma-detection-kits/venorgem-onestep-2>)

Cell freezing

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

Proceed as described for cell passaging. After resuspension of the cells in complete growth medium:

- Pellet cells by centrifugation (400 g, five minutes, RT).
- Work on ice for the following steps:
 - Resuspend 2×10^6 – 3×10^6 cells/cryotube in 0.5 mL/cryotube of FBS.
 - Mix with equal volume of freezing medium.
 - Transfer 1 mL per cryotube.
- Put the cryotubes in a freezing container (cell cooler) and store at -80°C for at least four hours.
- Transfer the tubes into liquid nitrogen vapor-phase storage tanks.
- Thaw one tube to test the batch.

Cell thawing

It is important to work quickly when thawing cells to wash out the DMSO (contained in the freezing medium) as rapidly as possible. Following thawing, passage the cells twice before using them to ensure consistent conditions.

To thaw the cells:

- Prepare 10 mL complete growth medium in centrifuge tubes.
- Warm the medium for 15 min in a 37°C water bath.
- Thaw the cells in a 37°C water bath until only a small amount of ice remains.
- Directly transfer the thawed cells into prepared complete growth medium.
- Pellet the cells by centrifugation (300 g, five minutes, RT).
- Discard the supernatant.
- Resuspend the cells in 15 mL complete growth medium and transfer them to a T75 flask.
- Maintain cells in culture as described in the 'HEp-2 cell passaging' section above.

3.3.4 Generation of viral stocks

Materials and reagents

The materials and reagents required are:

- See-saw rocker;
- Hep-2 cells;
- Virus dilution medium:
 - MEM (1x) + GlutaMAXTM (Gibco, 41090-028);
 - 100 U/mL Penicillin / 100 µg/mL Streptomycin (Pen/Strep) (Gibco, ref n° 15140148);
- Infection medium:
 - MEM (1x) + GlutaMAXTM (Gibco, 41090-028);
 - 2% FBS;
 - 100 U/mL Penicillin / 100 µg/mL Streptomycin (Pen/Strep) (Gibco, ref n° 15140148);
- 10x RSV cryoconservation solution:
 - 0.5M HEPES (SIGMA H3375);
 - 1M MgSO₄; pH 7.5;
 - Autoclaved, aliquoted and stored at -20°C or +4°C.

Method

Virus isolation from clinical PCR-positive specimens

RSV is isolated from clinical specimens for various purposes.

Clinical specimens used for virus isolation and characterisation may include nasopharyngeal swabs, saliva or bronchoalveolar lavage fluid. Fresh samples should be stored and transported at room temperature and inoculated whenever possible to optimise viral recovery. The efficacy of isolation decreases when the interval between collection and inoculation exceeds three days. When RSV is isolated from fresh samples, the efficacy of the isolation can be between 25% and 40% (unpublished data).

Samples previously frozen must be shipped at their original storage temperature to maintain stability and prevent freeze-thaw damage.

HEp-2 cells (seeded as indicated in Table 12) are preferred for RSV isolation and culture.

Preparation of cell plates

To prepare the cell plates:

- Plate 5×10^5 HEp-2 cells per well in 12-well plates a day in advance.

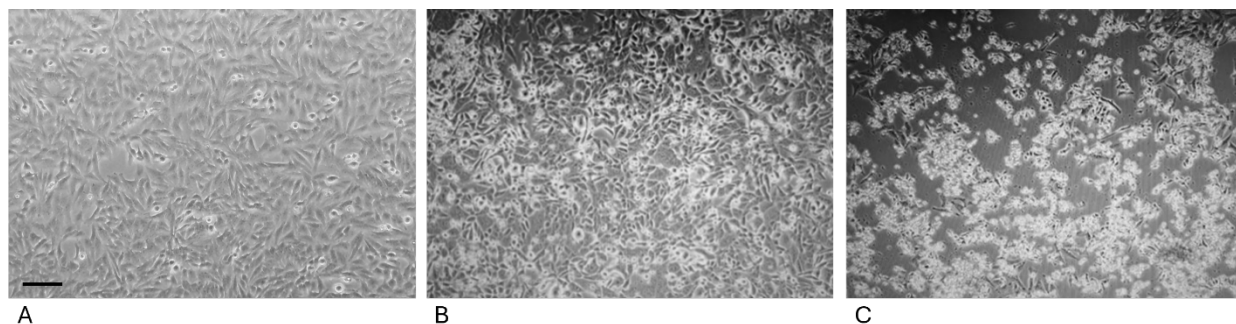
Cells should reach 90% confluence the day after.

Virus isolation

To isolate the virus:

- Remove the cell culture medium from the seeded 12-well plate.
- Add 100–150 µL of clinical specimen to the cells.
- Incubate the plate for two hours at 37°C in a 5%-CO₂ incubator with soft agitation on a see-saw rocker.
- Slowly add 1 mL of the infection medium to each well.
- Incubate the plate at 37°C in a 5%-CO₂ incubator for three to six days.
- Check the cells daily to monitor any cytopathic effect (CPE; syncytia formation, detachment of the cells).
- Harvest the cells and supernatant when CPE is massive (i.e. at least 80–90% of the cells show CPE, or on day six if there is still no massive CPE observed).

An example of cytopathic effect observed during the isolation process is presented in Figure 3.

Figure 3. Example of cytopathic effect observed during the isolation process

A: uninfected cells; B: mild cytopathic effect; C: massive cytopathic effect.
Scale bar: 20µm.

Harvesting

To perform harvesting:

- Detach the cells with a cell scraper.
- Transfer the cells and supernatant in a clean centrifuge tube.
- Vortex centrifuge tubes vigorously for at least 30 seconds to release viral particles from cell membranes.
- Add 1/10 volume of 10x RSV cryo-conservation solution.
- Vortex vigorously for five seconds.
- Quickly aliquot the samples into cryotubes at room temperature.
- Freeze the aliquots at -80°C, using pre-cooled ethanol at -80°C or an ethanol-dry ice bath. Avoid frozen nitrogen.
- Store at -80 °C.

Optional virus amplification step

If the cells don't show at least 50% CPE after six days, one may attempt to immediately amplify the isolated virus by infecting new HEp-2 cells seeded in a 12-well plate with 200µL of the fresh harvested virus (see 'Harvesting', above). The success rate of this second amplification is low (around 10%).

Generation of RSV stocks

Viral stocks are produced using a defined multiplicity of infection (MOI). Therefore, it is necessary to titrate the viral culture according to the method described in the plaque titration section beforehand.

Preparation of flasks

Plate HEp-2 cells in the appropriate flask the day before the infection, according to Table 12. The day of the infection, cells should have reached 90% confluence.

Stock production

Infect the HEp-2 cells as described below:

- Prepare the viral inoculum by diluting the viral stock in the virus dilution medium to reach an MOI of 0.005 plaque-forming units (PFU)/cell. If such a low MOI does not work, it can be increased to a range between 0.01 and 0.05.
- Discard the cell culture medium from the flask.
- Add the viral inoculum (see in Table 12 for the appropriate volume) onto the cells for adsorption.
- Incubate at 37°C in a 5%-CO₂ incubator for two hours on a see-saw rocker with soft agitation.
- Remove the viral inoculum and add the infection medium (volume in Table 12).
Incubate at 37°C in a 5%-CO₂ incubator for two to six days until 80–90% of the cells show CPE. The incubation time may vary depending on the virus. After ~3 dpi, 80–90% of the cells should be dead. Otherwise, cultivate cells one to three days longer before harvesting.

Harvesting

To conduct the harvesting:

- Add 1/10 of 10X RSV cryoconservation solution to the flask.
- Detach the cells with a cell scraper and transfer the cells with the supernatant to a centrifuge tube.
- Vortex vigorously for two minutes.
- Clarify by centrifugation (5 min, 400 g).
- Transfer the supernatant to a clean 50 mL centrifuge tube, vortex and aliquot.
- Quickly freeze the aliquots at -80°C (using ethanol at -80°C or an ethanol-dry ice bath, avoid frozen nitrogen).
- Store at -80°C.

Quality check the virus stocks

Finally, quality check the virus stocks:

- Titrate twice, as described in the 'Titration' section below, independently (use different aliquots, at least on two different days, and at least two different cell passages). The expected viral titre should be between 10^5 – 10^7 PFU/mL.
- Perform next generation sequencing (NGS) and check for mutations.

3.3.5 RSV plaque assays

Various plaque assays can be performed to test the viral titre of a culture or perform plaque reduction experiments.

Materials and reagents

The required materials and reagents are:

- HEp-2 cells;
- AVICEL RC591 (FMC Biopolymer); this product is not commercially available in small quantities, but FMC biopolymer is very helpful and 500 g samples can be obtained.
- Virus dilution medium:
 - MEM (1x) + GlutaMAX (Gibco, 41090-028);
 - 100 U/mL Penicillin / 100 µg/mL Streptomycin (Pen/Strep) (Gibco, ref n° 15140148);
- Infection medium:
 - MEM (1x) + GlutaMAX (Gibco, 41090-028);
 - 2% FBS;
 - 100 U/mL Penicillin / 100 µg/mL Streptomycin (Pen/Strep) (Gibco, ref n° 15140148).

MEM 2X solution can be prepared according to Table 14. The mixture should be shaken vigorously and can be stored at 4°C.

Crystal violet solution can be prepared according to Table 15. The mixture should not be exposed to light and can be stored at room temperature.

Table 14. MEM 2X solution

Components	Volumes
MEM 10x (Gibco, Ref 21430-020)	50 mL
L-Glutamine 0.2 M (Gibco, Ref 25030-024)	5 mL
100 U/mL Penicillin / 100 µg/mL Streptomycin (Pen/Strep) (Gibco, ref n° 15140148)	5 mL
Cell culture grade water	190 mL

Table 15. Crystal Violet solution

Components	Volumes
Crystal Violet Oxalate (RAL, Ref 3614963)	100 mL
Formaldehyde	100 mL
Ethanol Absolute	200 mL
MilliQ water	Up to 1 L

AVICEL RC591 (FMC Biopolymer) 2.4% solution can be prepared as follows:

- Disperse 2.4 g of AVICEL in 100 mL distilled water using a magnetic stirrer until complete dissolution (usually 4 to 12 hours).
- Autoclave the suspension at 121°C for 20 minutes.

Store at room temperature or 4°C before use. In such conditions, the suspension is stable for one year.

After opening the solution in a sterile environment, it can be stored at 4°C for six months. Always mix the suspension before use (by hand shaking or vortexing) to make sure it is homogeneous.

The same overlay solution is used for the different plaque assays (prepare around 33 mL per plate). The solution has to be prepared extemporaneously when performing the assay.

Overlay solution can be prepared according to Table 16. When preparing the overlay solution, adjust the pH of the MEM 2X solution by adding sodium bicarbonate 7.5% (Gibco, 25080-060) dropwise until the solution turns red.

Table 16. Overlay solution solution

Components	Volume
Infection medium	3:4 volume
Avicel solution 2.4%	1:8 volume
MEM 2X	1:8 volume

Method

RSV Plaque titration assay

This protocol describes the method to determine the titre of RSV stocks expressed in plaque-forming units (PFU)/mL [17].

Preparation of cell plates

Plate 5×10^5 HEp-2 cells per well in 12-well plates a day in advance. Cells should reach 90% confluence the day after.

Titration

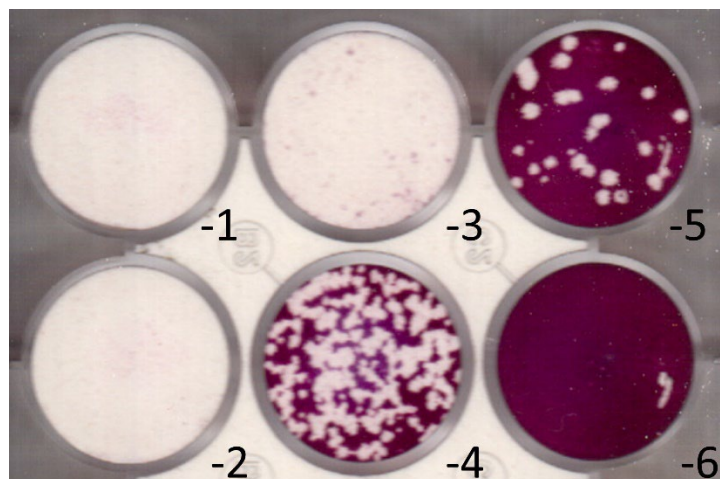
To conduct the titration:

- Prepare a series of tenfold virus dilutions (10^{-1} to 10^{-6}) in MEM 1x supplemented with 100 U/mL Pen/100 µg/mL Strep (virus dilution medium) by adding and mixing 100 µL of the previous dilution in 900 µL of virus dilution medium.
- Change filter pipette tips between each dilution.
- Discard the cell culture medium from the 12-well plate.
- Dispense 400 µL of each virus dilution into the wells. Do not process more than two plates at once, because the cells dry quickly under the biosafety cabinet.
- Incubate the plate for two hours at 37°C in a 5%-CO₂ incubator for virus adsorption.
- Prepare the overlay solution (around 2.5 mL per well).
- Warm the overlay at 37°C.
- After the two hours of adsorption, add 2–2.5 mL of the overlay solution to each well.
- Gently shake the plates to mix.
- Incubate for six days at 37°C in a 5%-CO₂ incubator. Do not move or shake the plates. The incubation period can be shortened to five days or extended to seven days. This may slightly alter the size of the plaques.

Revealing

To conduct the revealing:

- Gently shake the plate to loosen Avicel deposits.
- Discard the overlay.
- Rinse each well twice with PBS.
- Cover the cells with the Crystal Violet solution and incubate at room temperature for at least 10 minutes.
- Remove the Crystal Violet solution. Note that the Crystal Violet solution can be reused at least 10 times.
- Rinse each well with PBS or water to remove all the Crystal Violet solution.
- Disinfect the plates by plunging them into a bleach solution, then rinse them with clear water and dry them. Waste should be managed in accordance with your country's regulations and policies. Note that the plates can be recoloured even months later by repeating the Crystal Violet staining process.
- Count lysis plaques in the optimal dilution (dilution 10^{-5} in Figure 4) and calculate the titre: $\text{Titre} = (\text{number of plaques}) / (\text{volume inoculum} \times \text{dilution factor})$.

Figure 4. Example of a plaque titration assay

27 plaques at 10^{-5} dilution. Titre = $27 / (0.4 \times 10^{-5}) = 6.75 \times 10^6$ PFU/mL.

Testing RSV neutralisation by antibody (Nirsevimab) via plaque reduction assay

This protocol describes the method to test RSV's resistance to Nirsevimab.

Preparation of cell plates

The day before the assay, plate HEP-2 cells in 24-well plates according to Table 12. Cells should reach 90% confluency on the day of the assay.

Assay

To conduct the assay:

- Prepare a series of twofold dilutions of Nirsevimab in virus dilution medium, ranging from 100 ng/mL to 0.4 ng/mL (concentration of the dilutions), by adding and mixing 120 µL of the previous dilution in 120 µL of virus dilution medium. The final volume of each dilution is 120 µL.
- Prepare viral inoculums: 40–50 PFU/100 µL in virus dilution medium.
- Incubate the Nirsevimab dilutions with the viral inoculums (120 µL + 120 µL) for one hour at 37°C in a 5%-CO₂ incubator on a see-saw rocker for soft agitation.
- Discard the cell culture medium of the HEP-2 plates.
- Add 200 µL of the virus/Nirsevimab mixture to the cells and incubate them for two hours at 37°C in a 5%-CO₂ incubator for virus adsorption.
- Prepare the overlay solution (1–2 mL per well).
- Warm the overlay at 37°C.
- After the two hours of adsorption, add 1–2 mL of the overlay solution in each well.
- Incubate for six days at 37°C in a 5%-CO₂ incubator. Note that the incubation time depends on the virus and can be determined when performing the plaque titration assay.

Revealing

To conduct the revealing:

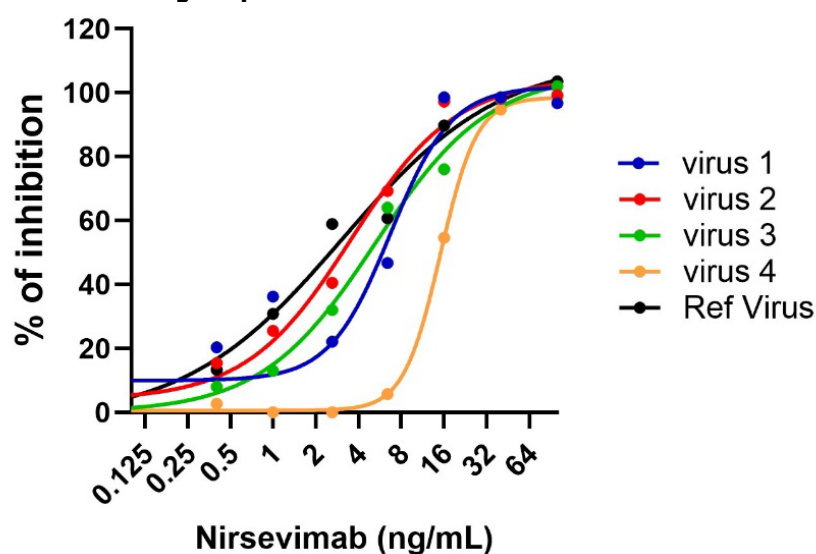
- Gently shake the plates to loosen Avicel deposits.
- Discard the overlay.
- Rinse each well twice with PBS.
- Cover the cells with the Crystal Violet solution and incubate them for at least 10 minutes at room temperature.
- Remove the Crystal Violet solution. Note that the Crystal Violet solution can be reused at least 10 times.
- Rinse each well with PBS or water to remove all the Crystal Violet.
- Disinfect the plates by plunging them into a bleach solution, then rinse them with clear water and dry them.

Determine the PFUs and report them to the Nirsevimab concentration in order to calculate the half maximal inhibitory concentration (IC₅₀). An example is provided in Figure 5.

You can use the GraphPad Prism software to run a regression analysis:

- Enter Nirsevimab concentration as x .
- Enter percentage of plaques as y (100%: number of plaques when Nirsevimab concentration equals zero).
- Run a nonlinear regression (CurveFit) analysis, with the inhibitor vs response (four parameters) setting.

Figure 5. Example of the results of calculating the half maximal inhibitory concentration (IC₅₀) of Nirsevimab using GraphPad Prism software



Sample	Nirsevimab	
	IC ₅₀ (ng/mL)	Fold Change IC ₅₀
Virus 1	6.7	2.2
Virus 2	3.5	1.2
Virus 3	5.3	1.7
Virus 4	15.1	4.9
Ref Virus	3.1	1.0

Testing human serum specimens to detect neutralising antibodies against RSV by plaque reduction assay

To test human serum specimens for neutralising antibodies against RSV, use a reference serum with a defined endpoint titre to ensure that the reagents are still working.

The assay can be calibrated with the first and second WHO International Standard for Antiserum to Respiratory Syncytial Virus (NIBSC 16/284 and 16/322).

Preparation of cell plates

The day before the assay, plate HEp-2 cells in 24-well plates, according to Table 12. Cells should reach 90% confluency on the day of the assay.

Assay

Conduct the assay as follows:

- Heat-inactivate the complement in sera at 56°C for 30 minutes in a water bath.
- Prepare a series of twofold dilutions of serum in virus dilution medium, ranging from 1:80 to 1:40960 – adapt to the titre of antibody in the serum.
- Prepare viral inoculums: 40–50 PFU/100µL in virus dilution medium.
- Mix the serum dilutions with the viral inoculums (120 µL + 120 µL) and proceed as indicated above for the Nirsevimab assay.
- Incubate for six days at 37°C in a 5%-CO₂ incubator. Note that the incubation time depends on the virus and can be determined when performing the plaque titration assay.

Revealing

Proceed as indicated for the Nirsevimab assay.

Determine the PFUs and report them to the serum dilutions in order to calculate the 50% effective dilution (ED₅₀). You can use the GraphPad Prism software to run a regression analysis:

- Enter serum dilution as x .
- Enter the percentage of plaques as y (100%: number of plaques when serum dilution equals zero). Run a nonlinear regression (CurveFit) analysis, with the inhibitor vs response (four parameters) setting.

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