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# Short amplicons panels (Artic-like) for RSVA and RSVB

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## ABSTRACT

This SOP describes the procedure for generating cDNA from respiratory syncytial virus A or B (RSVA or RSVB) viral nucleic acid extracts and subsequently producing ~400nt amplicons tiling the viral genome in a multiplex PCR.
The resulting products can be sequenced using short (Illumina) or long (Oxford Nanopore Technologies) reads approaches.
The panels and files for bioinformatic analysis are available at:
https://github.com/SimonLoriereLab/RSV_amplicons_panels

## GUIDELINES

The panels have been optimized using RSV A or RSV B positive samples circulating in Europe in 2018-
2022. Processing samples from previous years or from regions outside of Europe might require
further adjustments.

**Protocol status:** Working
We use this protocol and it's working

**Created:** Nov 07, 2023

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**Keywords:** rsv sequencing, amplicon, panel, RSV, RSVA, RSVB, Respiratory syncytial virus, NGS, HTS, Illumina, ONT, Oxford Nanopore Technologies

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## MATERIALS

### Primers

Primer schemes for RSVA and RSVB have been designed and optimized to cover the diversity of viruses circulating in the Northern hemisphere (2018-2023). Processing samples from previous years or from regions outside of Europe might require further adjustments. See the file below for primer sequences and their ratio.

We work with typed samples, but it may be possible to combine the panels for A and B.
Multiple assays are available for typing:
(1) Todd A et al, J Virol Methods, 2021 - Rapid detection of human respiratory syncytial virus A and B by duplex real-time RT-PCR. doi:10.1016/j.jviromet.2021.114171
(2) Wang et al, J Virol Methods, 2019 - Duplex real-time RT-PCR assay for detection and subgroup-specific identification of human respiratory syncytial virus. doi: 10.1016/j.jviromet.2019.113676

Other panels, including a panel compatible with both RSVA and RSVB have been developed:
Dong et al, J Clin Virol, 2023 - A simplified, amplicon-based method for whole genome sequencing of human respiratory syncytial viruses. doi: 10.1016/j.jcv.2023.105423

Primers may be ordered from any oligonucleotide company. For primer preparation instructions see **Step 5** of the protocol.

### Consumables

Filtered pipette tips
1.5 and 2mL microcentrifuge tubes
15 and 50 mL Falcon tubes
8-strip PCR tubes or PCR plates with caps or heat-sealing film
Reservoirs
Waste containers
Magnetic rack for PCR tubes or 96w plates

### Molecular Biology

LunaScript RT SuperMix, M3010X, NEB - or equivalent
Q5 High Fidelity DNA polymerase, M0491L, NEB
dNTP Mix 10MM, 10319879, Thermo Fisher Scientific
Agencourt AMPure beads XP, A63880, Beckman Coulter
Nuclease Free Water, 10526945, Thermo Fisher Scientific
Qubit dsDNA HS Assay kit, Q32854, Thermo Fisher Scientific

PROTOCOL MATERIALS

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Step 16

Agencourt AmPure XP beads **Catalog #A63880**

Step 15.1

SAFETY WARNINGS

Wear appropriate personal protective equipment.

BEFORE START INSTRUCTIONS

It is recommended to use to the forward flow principle to avoid contamination. All mastermixes should be made in a **mastermix cabinet** and aliquoted . Samples should be added in the **extraction and sample cabinet.** Amplicons should be purified in **a post-PCR cabinet**.
**Mastermix, extraction and sample** and **post-PCR cabinet** should be cleaned with appropriate disinfectant and UV-sterilized before and after use.

## RNA preparation

1 **Sample selection:**
Viral RNA input from a clinical sample (recommended Ct value below 28).

It is recommended to dilute high viral load samples (below Ct 14-15) to reduce the likelihood of PCR inhibition.

2 **Viral RNA extraction:**
Viral RNA should be extracted with QIAamp Viral RNA extraction kit (Qiagen) or equivalent, following the manufacturer instructions.

Note

*This step is also compatible with NucleoSpin 8x48 virus Core kit (Macherey-Nagel)*

## cDNA preparation

3 In a **mastermix cabinet**, mix the following components:

| Component | Volume |
| --- | --- |
| LunaScript Master Mix | 2 µL |
| Template RNA | 8 µL |

Mix by pipetting gently and spin down the tube.

Note

**Always add a no-template RT-PCR control** with nuclease-free water.

*Most cDNA kits should work for this step (also tested with SuperScript III or IV (Invitrogen))*

4 Incubate as follows: 23m

25 °C for 00:02:00
55 °C for 00:20:00
95 °C for 00:01:00
Hold at 4 °C
Then, place On ice .

## Primer pool preparation

5 Resuspend lyophilized primers at a concentration of 100µM each.

Note

Primer schemes for RSVA and RSVB have been designed and optimized to cover the diversity of viruses circulating in the Northern hemisphere (2018-2023). They generate overlapping ~400bp amplicons.
See the file below for primer sequences and their ratio.

6 In **a mastermix cabinet**, generate working primer stocks by diluting primers at the adequate concentration in nuclease-free water (for example in a 96-well plate).
Note that some primers need to be added at a different concentration to help normalize sequencing coverage.

7

For each RSV subtype, prepare both primer pool stocks by adding 5 µL of each primer pair to a 1.5 mL Eppendorf tube labelled either RSV A Pool 1, RSV A Pool 2, RSV B Pool 1 or RSV B Pool 2.

Note

**RSV A:**
Pool 1 has **50** primers, Pool 2 has **50** primers.
By adding 5 µL, total volume should be 250 µL for Pool 1 and 250 µL for Pool 2.

**RSV B:**
Pool 1 has **52** primers, Pool 2 has **54** primers.
By adding 5 µL, total volume should be 260 µL for Pool 1 and 270 µL for Pool 2.

8 Dilute the primer pool stocks to 1:10 in nuclease-free water to generate working primer pool stocks.

Note

Make aliquots of both primer pools stocks in case of degradation or contamination. Store at -20 °C .
Primers need to be used at a final concentration of [M] 0.015 micromolar (µM) per primer.

## Multiplex PCR

9 In a **mastermix cabinet**, set up the multiplex PCR reactions as follows:

**>RSV A:**

| **Component** | **Pool 1 or Pool 2** |
|---|---|
| 5X Q5 reaction buffer | 5 µL |
| 10mM dNTP | 0.5 µL |
| Primer Pool 1 **or** 2 (working stock) | 1.88 µL |
| Nuclease-free Water | 14.9 µL |

| | |
|---|---|
| Q5 Polymerase | 0.25 µL |
| **Total** | 22.5 µL |

**>RSV B:**

| **Component** | **Pool 1** | **Pool 2** |
|---|---|---|
| 5X Q5 reaction buffer | 5 µL | 5 µL |
| 10mM dNTP | 0.5 µL | 0.5 µL |
| Primer Pool 1 **or** 2 (working stock) | 1.95 µL | 2.02 µL |
| Nuclease Free Water | 14.73 µL | 14.8 µL |
| Q5 Polymerase | 0.25 µL | 0.25 µL |
| **Total** | 22.5 µL | 22.5 µL |

It is recommended to prepare a mastermix corresponding to the number of samples to be processed, always including a no template control per reaction.

Note: This highly multiplex reaction has been designed and tested with the NEB Q5 enzyme. Other types of enzymes may not be compatible.

10 In the **extraction and sample addition cabinet,** add 2.5 µL of cDNA to each tube and mix well by pipetting.

> **Note**
>
> Add a negative RT-PCR control with nuclease free water.

11 Spin down the tubes/strips/plates.

12 Set-up the following program on a thermal cycler: 5m 45s

| **Steps** | **Temperature** | **Time** | **Cycles** |
|---|---|---|---|
| Heat Activation | 98 °C | 00:00:30 | 1 |
| Denaturation | 98 °C | 00:00:15 | 35 |
| Annealing | 65 °C | 00:05:00 | 35 |

| | | | |
|---|---|---|---|
| Hold | 16 °C | infinite | 1 |

## PCR clean-up

13 *The following steps should be performed in a **post-PCR cabinet**.*

Combine the entire contents of Pool 1 and Pool 2 PCR reactions for each biological sample into a single tube.

14 Run 5 µL of each pooled sample on a 1% agarose gel.

**Expected result**

Expected amplicon size is ~400bp

15 Clean-up the amplicons using the following protocol.

15.1 **Vortex** SPRI beads (at room temperature) thoroughly. The solution should be homogenous if well resuspended.

Agencourt AmPure XP beads **Contributed by users** **Catalog #A63880**

15.2 Add 1,8X of SPRI beads ( 45 µL if no PCR product was run on a gel) to the sample tube and mix gently by pipetting.

15.3 Spin down the tubes to collect all liquid at the bottom of the tube.

15.4 Incubate 00:05:00 to 00:10:00 at Room temperature 15m

15.5 Place the tubes on an appropriate magnetic rack and incubate for 00:05:00 to 00:10:00 15m until the beads have pelleted and the supernatant is clear.

15.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

15.7 Add 200 µL of **fresh**, Room temperature 80% ethanol to the pellet while in the magnetic 1m rack, and incubate for 00:01:00 .

15.8 Carefully remove and discard ethanol, being careful not to touch the beads pellet.

15.9 go to step #15.7 and repeat ethanol wash.

15.10 Spin down the tubes to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette. Do not touch the beads.

15.11 Incubate the tubes for 00:05:00 at Room temperature with the lid open (drying step). 5m

15.12 Add 20 µL of nuclease-free water, remove the tubes from the magnetic rack and resuspend th 2m pellet by gently pipetting. Incubate for 00:02:00 .

15.13 Place the tubes back on the magnetic rack until the solution is clear and transfer the supernatant into clean tubes (e.g. 0.2mL 8-strip PCR). Make sure not to transfer any beads.

## Quantification and normalisation

16 Quantify the DNA concentration using the Qubit High Sensitivity DNA kit (or equivalent) from 1 µL of each product.

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

**Expected result**

Expected range = 10-100 ng/µL DNA.

## Library preparation

17 The PCR products can be used to prepare libraries for short or long reads sequencing.