IVD

Instructions for use (English)

1 Purpose

The *ampli*Cube Respiratory Viral Panel 5 is a qualitative *in-vitro* test for specific detection of the RNA of influenza A virus, influenza B virus and respiratory syncytial viruses A and B (RSV A/B) in human sputum, swabs, BAL (broncho-alveolar lavage) or tracheal secretions.

2 Field of application

Influenza A virus and influenza B virus are pathogens that cause seasonal influenza (flu) and belong to the Orthomyxoviridae family. Human respiratory syncytial viruses A and B are part of the Pneumoviridae family. The viruses can cause symptoms in the upper respiratory tract in particular. In small children and particularly in infants RSV infections often cause severe illness that may require hospitalisation for treatment. Other important viral respiratory pathogens such as rhinoviruses and human metapneumoviruses can induce very similar clinical symptoms. In terms of sensitivity and specificity, genetic characterisation of influenza is considered the gold standard.

3 Test principle

The test is a real-time RT (reverse transcriptase) PCR system. It uses specific primers and labelled probes for transcription of RNA into cDNA, amplification and then detection of the RNA of the influenza A virus, influenza B virus and RSV (A/B).
To ensure that the nucleic acids isolated from the patient sample do not contain any substances that inhibit RT-PCR, an internal control (IC) is added to the sample during the nucleic acid isolation. This IC is transcribed into cDNA, amplified and detected in the same RT-PCR batch. This eliminates false negative test results due to inhibition of the RT-PCR reaction. At the same time, the IC serves as evidence of nucleic acid extraction from the patient sample.
Probes to specifically detect the pathogen-specific nucleic acids are marked with the reporter dyes FAM (influenza A virus), HEX (influenza B virus) and ATTO Rho12 (RSV A/B) and the probes for detecting the internal control are marked with ATTO 647N. This allows simultaneous detection of all target sequences in a single reaction mixture.
The Ct value *(cycle threshold)* describes the part of the curve in which the fluorescence rises exponentially above the background value for the first time.

4 Reagents

4.1 Package contents

The reagents in one pack are sufficient for 50 assays.
Each set of reagents contains:

P&P MIX	**150 µl** primer & probe mix for Respiratory Viral Panel 5 and internal control (**green lid**)
ENZYME	**600 µl** enzyme mix **(white lid)**; contains reverse transcriptase and DNA polymerase (**component is stained blue**)
CONTROL INT	**250 µl** internal control (**transparent lid**); across kits and batches of all *ampli*Cube Respiratory Viral Panels and the Coronavirus Panel
CONTROL +	**170 µl** positive control (**red lid**)
CONTROL -	**2 × 1800 µl** negative control (**blue lid**); across kits and batches of all *ampli*Cube Respiratory Bacterial Panels, Respiratory Viral Panels and the Coronavirus Panel
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4.2 Additionally required reagents, materials and equipment

- MIKROGEN *ampli*Cube Color Compensation for LightCycler® 480 II (Roche)
- Commercial nucleic acid isolation kit. The following nucleic acid extraction system is recommended:
  MagNAPure® Compact, Total Nucleic Acid Kit I (Roche)
- Real-Time Cycler. The following cycler is recommended:
  LightCycler® 480 II (Roche)
- 96 well PCR plates and films or reaction tubes (PCR-clean), depending on the cycler
- Micropipettes with single-use tips with filter 10 µl, 20 µl, 100 µl and 1000 µl
- Vortex mixer
- Mini centrifuge
- If necessary, plate centrifuge
- Single-use gloves, powder-free
- Cooling block

5 Shelf life and handling

- Store reagents between -25 °C and -18 °C before and after use.
- Repeated thawing and freezing of the components (more than ten times) must be avoided. It is recommended to aliquot the test components after the first thawing.
- Always appropriately chill reagents during the working steps (+2 °C to +8 °C).
- Keep the kit components away from direct sunlight throughout the test procedure.
- Before starting the test, all reagents must be completely thawed, mixed (briefly vortex) and centrifuged.
- The packages have an expiry date, after which no further guarantee of quality can be given.
- The test must only be performed by trained, authorised and qualified personnel.
- Substantial changes made by the user to the product or the directions for use may compromise the intended purpose of the test specified by MIKROGEN.
- Cross-contamination can lead to false test results. Add patient samples and controls carefully. Ensure that the reaction mixtures are not transferred to other wells.
- The internal control (IC) included in this kit can be used across kits and batches for all *ampli*Cube Respiratory Viral Panels and the *ampli*Cube Coronavirus Panel. Note the shelf life when doing so.
- The negative control (NC) can be used across kits and batches for all *ampli*Cube Respiratory Bacterial Panels, *ampli*Cube Respiratory Viral Panels and the *ampli*Cube Coronavirus Panel. Note the shelf life when doing so.

6 Warnings and safety precautions

- Only use for *in-vitro* diagnostics.
- All patient samples must be treated as potentially infectious.
- Suitable single-use gloves must be worn throughout the entire test procedure.
- All reagents and materials that come into contact with potentially infectious samples must be treated with suitable disinfectants or be disposed of according to laboratory guidelines. The concentration specifications and incubation times of the manufacturer must be followed.
- Do not replace or mix the reagents with reagents from other kit batches or other MIKROGEN *ampli*Cube PCR kits, if they are not specifically intended for this kit. Do not replace or mix with reagents from other manufacturers.
- Read through and carefully follow all instructions before performing the test. Deviations from the test protocol described in the instructions for use can lead to false results.

7 Sampling and preparation of reagents

7.1 Sample material

The starting material for the *ampli*Cube Respiratory Viral Panel 5 is RNA extracted from sputum, swab, BAL or tracheal secretion of human origin. The quality of the nucleic acid preparation affects the test result. It must be ensured that the extraction method selected is compatible with the real-time PCR technology.

7.2 Extraction of nucleic acids

Extract the nucleic acids from the patient sample and the negative control (NC). We recommend a starting volume for the extraction of 200 µl and an elution volume of 50 µl. Extractions from 400 µl starting material eluted into 100 µl showed comparable results. Follow the instructions from the manufacturer of the extraction kit.

If you would like to analyse the eluate of the extraction parallel to the *ampli*Cube Respiratory Viral Panel using the *ampli*Cube Respiratory Bacterial Panel kits, please note the different use of the internal control. Appropriate directions can be found in the instructions for use of the *ampli*Cube Respiratory Bacterial Panel kits.

1. Thaw the internal control (IC) (transparent lid) and the negative control (NC) (blue lid).
   Ensure that the IC and the NC are completely thawed. Mix the IC and the NC before use by briefly vortexing and then briefly centrifuge.
2. For the extraction, add 5 µl (for 50 µl eluate) IC to each patient sample and the NC. The IC should be added to the sample/lysis buffer mix and not directly to the sample material.
3. Extract the patient sample and the NC. (Note: the NC cannot be used in the PCR without extraction.)
4. The positive control is not extracted.

The following nucleic acid extraction system is recommended and was used for the performance assessment:

Extraction system	Sample volume	Elution volume
MagNAPure® Compact (Roche) Total Nucleic Acid Kit I	200 µl	50 µl

If you would prefer to use other extraction methods, please contact the manufacturer to clarify the compatibility.

7.3 Preparing the master mix

1. Thaw the primer & probe mix (green lid) and the enzyme mix (white lid). Protect the reagents from light when doing so.
   Ensure that the reagents are completely thawed. Mix the reagents before use by briefly vortexing and then briefly centrifuge.
2. Prepare the master mix using the following pipetting scheme:

Components	Master mix for 1 reaction
Primer & probe mix	3 µl
Enzyme mix	12 µl
Total volume	15 µl

3. Mix the entire master mix by vortexing and then briefly centrifuge.
4. Produce 15 µl master mix for each PCR reaction.

7.4 Preparing the PCR reaction

1. Thaw the positive control (PC) (red lid).
   Ensure that the reagents are completely thawed. Mix the reagents before use by briefly vortexing and then briefly centrifuge.

Components	1 reaction
Master mix from 7.3	15 µl
Sample eluate or eluate of the NC or the PC	10 µl

2. Pipette 10 µl of the sample eluate into 15 µl of the master mix.
3. Pipette 10 µl of the positive control (not prepared) into 15 µl of the master mix.
4. Pipette 10 µl of the eluate of the negative control into 15 µl of the master mix.

Every run must include a positive and a negative control.
Seal the PCR plate with an adhesive optically clear film or seal the reaction tube with the lid provided.

⚠ **PCR plates and tubes must be vortexed for at least 10 sec at maximum speed, followed by brief centrifugation.**

8 Programming the real-time cycler

The *ampli*Cube Respiratory Viral Panel 5 was evaluated with the LightCycler® 480 Instrument II (Roche).

8.1 Setting the detection channels

	Influenza A virus	Influenza B virus	RSV (A/B)	Internal control (IC)
Channel	FAM	HEX	ROX	Cy5
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N
Colour	Green	Yellow	Orange	Red
Emission	510 nm	580 nm	610 nm	660 nm
Quencher	[none]	[none]	[none]	[none]

Details of the wavelengths for the detection channels refer to the LightCycler® 480 II.
For the LightCycler® 480 II, it is necessary to first use colour compensation, which is provided by MIKROGEN.

8.2 PCR program

Reverse transcription	50 °C	8 minutes
Denaturation	95 °C	3 minutes
Amplification	**45 cycles**	
• Denaturation	95 °C	10 seconds
• Annealing/elongation	60 °C	45 seconds

Essential information about programming the different real-time cyclers can be found in the instructions for the cycler used. For specific information about programming the real-time PCR cycler when using the *ampli*Cube Respiratory Viral Panel 5, please contact the manufacturer.

9 Results

9.1 Validation

1. The negative control must be below the *threshold*. The internal control (IC) must have a positive curve for the negative control. If the negative control has a positive curve (contamination) or if the IC is not valid in the negative control, the test run cannot be analysed.
2. The positive control must have a positive curve. The Ct value for the positive control must be < 33. A positive control outside this range indicates there is a problem with the amplification.
3. The internal control (IC) must have a positive curve for negative samples.
   The signal for the IC for a patient sample must be compared to the signal of the IC in the extracted negative control. A difference of > 3 Ct for the IC between the sample and the negative control or the absence of an IC signal for the sample may indicate significant inhibition of the RT-PCR reaction. In these cases a negative test result is invalid.

9.2 Evaluation

The data can be analysed with the corresponding PCR cycler software or a software solution for automated PCR analysis and interpretation specifically supported by MIKROGEN. When using a LightCycler® 480 II, analysis can be carried out either by *Abs Quant/2nd Derivative Max* method (recommended) or by *Abs Quant/Fit Points* method. Additional information and corresponding instructions are available from MIKROGEN upon request.

Signals above the *threshold* are evaluated as positive results. Empty fields in the table are considered negative results.

	Influenza A virus	Influenza B virus	RSV (A/B)	Internal control (IC)
Colour				
Green	Positive			
Yellow		Positive		
Orange			Positive	
Red				Positive*

*In the case of positive signals in the detection channels for the pathogens, the signal for the internal control is not required for the test interpretation. A high pathogen load in the patient sample can lead to a reduced or missing signal for the internal control.

10 Limits of the method, restrictions

- Test results must always be viewed in the context of the clinical situation. Therapeutic consequences of the findings must be linked to the clinical data.
- A negative influenza A virus, influenza B virus and RSV (A/B) test result cannot rule out infection with the particular pathogens.

11 Performance characteristics

11.1 Diagnostic sensitivity and specificity

The sensitivity and specificity were determined using defined positive and defined negative samples.

Table 1: Defined positive samples

*ampli*Cube Respiratory Viral Panel 5	**Influenza A virus** (n=20)	**Influenza B virus** (n=20)	**RSV (A/B)** (n=20)
Negative	0	0	0
Positive	20	20	20
Sensitivity	**100 %**	**100 %**	**100 %**

Table 2: Defined negative samples

*ampli*Cube Respiratory Viral Panel 5	**Influenza A virus** (n=20)	**Influenza B virus** (n=20)	**RSV (A/B)** (n=20)
Negative	20	20	20
Positive	0	0	0
Specificity	**100 %**	**100 %**	**100 %**

11.2 Analytical sensitivity

The limit of detection (LoD) of the *ampli*Cube Respiratory Viral Panel 5 was determined using a dilution series of plasmid DNA of known concentration in a LightCycler® 480 II System (Roche). The 95% limit of detection was determined using probit analysis with CombiStats™ Version 5.0 software (Council of Europe).

Table 3: Limit of detection (LoD)

	Influenza A virus	Influenza B virus	RSV (A/B)
LoD 95% limit of detection Genomes/PCR	5.34 (3.03 – 14.85)	3.10 (1.63 – 8.90)	1.98 (1.33 – 3.90)

11.3 Analytical specificity

The BLAST trial (www.ncbi.nlm.nih.gov/blast/) shows that the selected primers and probes of the *ampli*Cube Respiratory Viral Panel 5 specifically detect the selected pathogens.
Furthermore, the specificity was determined by studying genomic DNA/RNA of other human pathogenic bacteria and viruses.

Table 4: Bacteria and viruses that were tested to demonstrate the analytical specificity of the *ampli*Cube Respiratory Viral Panel 5.

Bacteria
Bordetella holmesii
Bordetella parapertussis
Bordetella pertussis
Chlamydia pneumoniae
Escherichia coli
Haemophilus influenzae
Klebsiella pneumoniae
Legionella pneumoniae
Moraxella catarrhalis
Mycoplasma pneumoniae
Pseudomonas aeruginosa
Staphylococcus aureus
Streptococcus pneumoniae

Viruses
Adenovirus A
Adenovirus Serotype 1 (C)
Adenovirus Serotype 3 (B)
Bocavirus
Coronavirus 229 E
Coronavirus HKU1
Coronavirus MERS
Coronavirus NL63
Coronavirus OC43
Coxsackievirus
Cytomegalovirus
Enterovirus 68
Epstein-Barr virus
Measles virus
Mumps virus
Parainfluenza 1
Parainfluenza 2
Parainfluenza 3
Parainfluenza 4

None of these samples generated a positive signal. The primers and probes used in the *ampli*Cube Respiratory Viral Panel 5 showed no cross-reactions with the pathogens listed in Table 4. The internal control (IC) was valid in all tests.

11.4 Equivalence of different sample material

The coefficient of variation (CV) was determined for the Ct value between water and the extract of the particular sample material after the addition of plasmid DNA of known concentration.

Table 5: Equivalence of different sample material

	Influenza A virus	Influenza B virus	RSV (A/B)
CV [%] (BAL, H_2O)	1.06	1.44	1.14
CV [%] (sputum, H_2O)	2.16	1.44	2.77
CV [%] (swab, H_2O)	1.16	0.75	0.89

The coefficient of variation (CV), based on the Ct value *(cycle threshold)* between water and the DNA extracts (obtained for the various sample materials), was ≤2.77 % for all target genes.

12 Literature

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3. Daniela Huzly et. al. (2016): Influenza A virus drift variants reduced the detection sensitivity of a commercial multiplex nucleic acid amplification assay in the season 2014/15. Arch Virol, June 2016, DOI 10.1007/s00705-016-2930-8
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11. John S. Tregoning et. al. (2010): Respiratory Viral Infections in Infants: Causes, Clinical Symptoms, Virology, and Immunology. Clinical Microbiology Reviews, Jan. 2010, Vol. 23, No. 1, p. 74-98, doi:10.1128/CMR.00032-09

We will be pleased to send you additional literature on request.

13 Explanation of symbols

Symbol	Meaning
Σ	Content is sufficient for <n> formulations Number of formulations
P&P MIX	Primer & probe mix
ENZYME	Enzyme mix
CONTROL INT	Internal control
CONTROL +	Positive control
CONTROL -	Negative control
INSTRU	Instructions for use
i	Follow the instructions for use
CONT	Contents, contains
IVD	*In-vitro* diagnostic agent
LOT	Batch/version number
REF	Order number
	Use by Expiry date
x°C y°C	Store between x °C and y °C
	Manufacturer

14 Manufacturer and version data

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