*ampli*Cube Respiratory Viral Panel 4

MIKROGEN DIAGNOSTIK

IVD

Instructions for use (English)

1 Purpose

The *ampli*Cube Respiratory Viral Panel 4 is a qualitative in-vitro test for specific detection of the RNA of rhinovirus / enterovirus and the DNA of adenovirus (A-G) in human sputum, swabs, BAL (broncho-alveolar lavage) or tracheal secretions.

2 Field of Application

Rhinoviruses are members of the *Enterovirus* genus in the Picornaviridae family. Even though rhinoviruses are often underestimated because of their milder clinical symptoms, together with the respiratory enteroviruses, they are the pathogens that most commonly cause acute infections in the upper respiratory tract. The clinical symptoms range from asymptomatic infections to infections of the respiratory tract to a disseminated infection. Human adenoviruses are human pathogenic viruses from the Adenoviridae family and, depending on the species (A–G), can cause a range of diseases. Adenoviruses primarily cause disease of the respiratory tract and they are detected in about 5% of acute respiratory diseases in children. Patients with compromised immune systems are particularly susceptible to serious complications such as *acute respiratory distress syndrome*. The *ampli*Cube Respiratory Viral Panel 4 allows these viral pathogens to be reliably detected.

3 Test Principle

The test is a real-time RT-PCR system. It uses specific primers and marked probes for amplifying and detecting RNA from rhinovirus / enterovirus and adenovirus.
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 RNA isolation. This IC is reverse transcribed, amplified, and detected in the same RT-PCR reaction. This enables false negative test results due to inhibition of the RT-PCR reaction to be ruled out. At the same time, the IC serves as evidence of nucleic acid extraction from the patient sample.
Probes for detecting pathogen-specific RNA are marked with the reporter dyes FAM (rhinovirus / enterovirus), and ATTO Rho12 (adenovirus) 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 of one pack are sufficient for 50 assays.
Each set of reagents contains:

P&P MIX	**150 µl** primer & probe mix for Respiratory Viral Panel 4 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 (**colourless lid**)
CONTROL +	**170 µl** positive control (**red lid**)
CONTROL -	**2 × 1800 µl** negative control (**blue lid**)
INSTRU	**1** instructions for use

4.2 Additionally required reagents, materials and equipment

- MIKROGEN *ampli*Cube Color Compensation for Light Cycler® 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:
  Light Cycler® 480 II (Roche)
- 96 well PCR plates and films or reaction tubes (PCR-clean), dependent on the cycler
- Micropipettes with single-use tips with filter 10 µl, 20 µl, 100 µl and 1000 µl
- Vortex mixer
- Mini centrifuge
- Possibly 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 – +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.

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 coming 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 taken into consideration.
- Do not replace or mix the reagents with reagents from other kit batches, other MIKROGEN PCR kits or 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 Reagent Preparation

7.1 Sample material

The starting material for the *ampli*Cube Respiratory Viral Panel 4 is DNA and 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.

1. Thaw the internal control (IC) (colourless 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. (Note: the IC cannot be used in the PCR without extraction!)
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 while 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. Introduce 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 the master mix.
3. Pipette 10 µl of the positive control (not prepared) into the master mix.
4. Pipette 10 µl of the eluate of the negative control into the master mix.

Every run must include a positive and a negative control.
Seal the PCR plate with an adhesive optically clear film or 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 4 was evaluated with the LightCycler® 480 Instrument II (Roche).

8.1 Setting the detection channels

	Rhinovirus / Enterovirus	Adenovirus	Internal control (IC)
Reporter dye	FAM	ATTO Rho12	ATTO 647N
Colour	Green	Orange	Red
Emission	510 nm	610 nm	660 nm
Quencher	[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 4, please contact the manufacturer.

9 Results

The data analysis for the LightCycler® 480 II uses the *Abs Quant/2nd Derivative Max* method.

9.1 Validation

1. The negative control must be below the *threshold*. If this control is contaminated (positive curve), the test run cannot be evaluated.
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 must have a positive curve for negative samples and the negative control. A deviation in the curve progression of the IC in negative samples in comparison with the negative control indicates that there is a problem in the extraction of the nucleic acids or inhibition of the RT-PCR.

9.2 Evaluation

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

	Rhinovirus / Enterovirus	Adenovirus	Internal control (IC)
Colour			
Green	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 Limitations and Restrictions of the Method

- 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 rhinovirus / enterovirus and/or adenovirus test result cannot rule out an infection with the particular pathogen.

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 4	Rhinovirus / Enterovirus (n=37)	Adenovirus (n=16)
Negative	0	0
Positive	37	16
Sensitivity	**100%**	**100%**

Table 2: Defined negative samples

*ampli*Cube Respiratory Viral Panel 4	Rhinovirus / Enterovirus (n=24)	Adenovirus (n=24)
Negative	24	24
Positive	0	0
Specificity	**100%**	**100%**

11.2 Analytical sensitivity

The limit of detection (LoD) of the *ampli*Cube Respiratory Viral Panel 4 was determined using a dilution series of plasmid DNA of known concentration on 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)

	Rhinovirus / Enterovirus	Adenovirus
LoD 95% limit of detection Genome/PCR	14.83* (8.84 – 37.06)	40,44 (21.23 – 137.12)

*Data refer to rhinovirus B; furthermore rhinovirus A, C, Coxsackie virus and echovirus were tested. The detection limits were between 25.51 and 35.93.

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 4 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 4.

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

Viruses
Coronavirus 229 E
Coronavirus HKU1
Coronavirus MERS
Coronavirus NL63
Coronavirus OC43
Cytomegalievirus
Epstein-Barr virus
Human metapneumovirus A
Measles virus
Mumps virus
Parainfluenza 1
Parainfluenza 2
Parainfluenza 3
Parainfluenza 4
Paechovirus
Respiratory syncytial virus A
Respiratory syncytial virus B

None of these samples generated a positive signal. The primers and probes used in the *ampli*Cube Respiratory Viral Panel 4 showed no cross-reaction 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

	Rhinovirus / Enterovirus	**Adenovirus**
CV [%] (BAL, H_2O)	0.69	0.59
CV [%] (sputum, H_2O)	1.66	1.34
CV [%] (swab, H_2O)	0.44	1.06

The coefficient of variation (CV), based on the Ct value (*cycle threshold*) between water and the nucleic acid extracts (obtained for the various sample materials), was ≤ 1.66% for all target genes.

12 Literature

1. M. Damen et.al. (2008): Real-Time PCR with an Internal Control for Detection of All Known Human Adenovirus Serotypes. Journal of Clinical Microbiology, Dec. 2008, p. 3997–4003
2. Albert Heim et al. (2003): Rapid and Quantitative Detection of Human Adenovirus DNA by Real-Time PCR. Journal of Medical Virology 70:228–239 (2003)
3. Jin Li et. al. (2013): A Two-Tube Multiplex Reverse Transcription PCR Assay for Simultaneous Detection of Sixteen Human Respiratory Virus Types/Subtypes, Hindawi Publishing Corporation BioMed Research International Volume 2013, Article ID 327620
4. Ozeas Galeno da rocha Neto et. al. (2013): Update on viral community-acquired pneumonia. Revista da Associacao Medica Brasileira, Sept. 2012, 59(1):78-84
5. Marta C. Nunes et. al. (2013): Clinical Epidemiology of Bocavirus, Rhinovirus, Two Polyomaviruses and Four Coronaviruses in HIV-Infected and HIV-Uninfected South African Children Respiratory Viruses in Hospitalized Children, February 2014, Volume 9, Issue 2
6. Andrew T. Pavia (2011): Viral Infections of the Lower Respiratory Tract: Old Viruses, New Viruses, and the Role of Diagnosis. Clinical Infectious Diseaes, 2011;52(S4):S284–S289
7. Fanny Renois et. al. (2010): Rapid Detection of Respiratory Tract Viral Infections and Coinfections in Patients with Influenza-Like Illnesses by Use of Reverse Transcription-PCR DNA Microarray Systems. Journal of Clinical Microbiology, Nov. 2010, Volume 48, No. 11, p. 3836-3842, doi:10.1128/JCM.00733-10
8. 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
9. Aimee K. Zaas et. al. (2009): Gene Expression Signatures Diagnose Influenza and Other Symptomatic Respiratory Viral Infection in Humans. Cell Host Microbe., Sept. 2009; 6(3): 207–217, doi:10.1016/j.chom.2009.07.006

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

13 Explanation of symbols

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
	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

***ampli*Cube Respiratory Viral Panel 4**	Article no. **50132**
Instructions for use Valid from	GAACRV4002EN 2023-04

MIKROGEN GmbH
Anna-Sigmund-Str. 10
82061 Neuried
Germany
Tel. +49 89 54801-0
Fax +49 89 54801-100
Email mikrogen@mikrogen.de
Internet www.mikrogen.de

CE

GAACRV4002