IVD

Instructions for use (English)

1 Intended purpose

The *ampli*Cube Coronavirus Panel is a qualitative in-vitro test to specifically detect the RNA of Middle East Respiratory Syndrome Coronavirus (MERS-CoV), the RNA of novel SARS-CoV-2, SARS-CoV and Bat SARS-like CoV, and the RNA of human coronaviruses (HCoV) (NL63, OC43, 229E, HKU1) in human sputum, swabs, BAL (bronchoalveolar lavage), tracheal secretions or stool.

2 Field of application

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and human coronaviruses (HCoV) including the NL63, OC43, 229E and HKU1 species are part of the *Coronaviridae* family and are important as the pathogens that cause mild respiratory infections to severe acute respiratory syndrome. About one-third of all colds are caused by HCoV with diarrhoea being a common attendant symptom. MERS-CoV is responsible for Middle East respiratory syndrome, a disease that often manifests as an acute, often severe, respiratory flu-like disease with pneumonia and respiratory distress. Differential diagnostics should consider MERS-CoV particularly if there has been contact with persons infected with MERS-CoV in one of the Middle Eastern countries in the 2 weeks prior to the start of the illness.
SARS coronaviruses such as SARS-CoV-2 (causative pathogen of the COVID-19 pandemic) spread primarily by transmission from person to person via droplets in exhaled air. Symptoms range from fever, cough and dyspnoea to pneumonia and acute respiratory distress syndrome and ultimately death in persons with comorbidities.
The *ampli*Cube Coronavirus Panel is used to detect and differentiate MERS-CoV, SARS-CoV (particularly SARS-CoV-2) and HCoV.

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 and amplification and detection of the RNA of MERS-CoV, SARS-CoV-2, SARS-CoV, Bat SARS-like CoV and HCoV (NL63, OC43, 229E, HKU1).
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 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 (MERS-CoV), HEX (SARS-CoV-2, SARS-CoV, Bat SARS-like CoV) and ATTO Rho12 (HCoV) 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 MERS-CoV, SARS-CoV, HCoV 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**)
CONTROL +	**170 µl** positive control (**red lid**)
CONTROL -	**2 x 1800 µl** negative control (**blue lid**)
INSTRU	**1** instructions for use

4.2 Additionally required reagents, materials and equipment

- Depending on the used real-time PCR-Cycler, MIKROGEN provides reagents for the colour compensation:
  MIKROGEN *ampli*Cube Color Compensation (LightCycler® 480 II (Roche) article no. 50502), MIKROGEN *ampli*Cube Color Compensation (cobas z 480 Analyzer (Roche), article no. 50503) or dye calibration kit for CFX96 (Bio-Rad, article no. 50505). If processing at Mic (bms) PCR cycler, MIKROGEN provides Mic assay-templates.
- Nucleic acid extraction: The following nucleic acid extraction systems are recommended: MagNA Pure® System, Total Nucleic Acid Isolation Kit (Roche) or *alpha*Clean Mag RNA/DNA Kit (MIKROGEN) with processing on the M32, M48 or M96 extractor (Biocomma)
- Real-time Cycler: LightCycler® 480 II (Roche), cobas z 480 Analyzer (Roche), CFX96 (Bio-Rad), QuantStudio 5 (Applied Biosystems), Mic (bms), Rotor-Gene Q (Qiagen)
- 96-well PCR plates and films or reaction tubes (PCR-clean): follow the recommendations of the manufacturer of the real-time PCR cycler
- Micropipettes with single-use tips with filter 10 µl, 20 µl, 100 µl and 1000 µl
- Vortex mixer with high rotational speed (recommended 3200 rpm)
- Mini centrifuge
- If necessary, plate centrifuge
- PBS or H_2O *(PCR grade)* if using flocked nylon fibre swabs with no transport medium
- 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.

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 concentrations and incubation times specified by the manufacturer must be followed.
- 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 preparation of reagents

7.1 Sample material

The starting material for the *ampli*Cube Coronavirus Panel is RNA extracted from sputum, swab, BAL, tracheal secretion or stool of hu-

man 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.

7.1.1 *Sample preparation*

When using a flocked nylon fibre swab without transport medium:

1. Incubate the swab in 0.5 ml PBS (for extraction using the MagNA Pure® system) or 0.5 ml H_2O (for extraction using *alpha*Clean Mag RNA/DNA) for 5 min at room temperature.
2. Discard the swab and use the PBS or H_2O suspension for the nucleic acid extraction.

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 or 100 µl depending on the extraction system. Extractions (MagNA Pure® System (Roche) 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) (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 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
MagNA Pure® 24 (Roche) Total NA Isolation Kit	200 µl	50 µl
M96 Nucleic Acid Extraction System (biocomma) *alpha*Clean Mag RNA/DNA Kit (MIKROGEN)	200 µl	100 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 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 RT-PCR reaction

1. Thaw the positive control (PC) (red lid).
   Ensure that the reagents are completely thawed. Mix the reagents before use by 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 master mix.
3. Pipette 10 µl of the positive control (not prepared) into 15 µl master mix.
4. Pipette 10 µl of the eluate of the negative control into 15 µl master mix.

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

⚠ The PCR plates or test tubes must be vortexed for <u>at least 5 sec</u> at maximum speed and then briefly centrifuged.

⚠ PCR test tubes for the <u>Mic PCR cycler</u> must be vortexed for <u>at least 10 sec</u> at maximum speed.

8 Programming the real-time cycler

The *ampli*Cube Coronavirus Panel was evaluated with the LightCycler® 480 Instrument II (Roche) and validated at cobas z 480 Analyzer (Roche), CFX96 (Bio-Rad), QuantStudio 5 (Applied Biosystems), Mic (bms) and Rotor-Gene Q (Qiagen).

8.1 Setting the detection channels

LightCycler® 480 Instrument II (Roche)

	MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour	Green	Yellow	Orange	Red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N
Excitation	465 nm	533 nm	533 nm	618 nm
Emission	510 nm	580 nm	610 nm	660 nm

For the LightCycler® 480 II, it is necessary to first use the Color Compensation kit (article no. 50502), which is provided by MIKROGEN.

cobas z 480 Analyzer (Roche)

	MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour	Green	Yellow	Orange	Red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N
Excitation	465 nm	540 nm	540 nm	610 nm
Emission	510 nm	580 nm	610 nm	670 nm

For the cobas z 480 Analyzer (Roche), it is necessary to first use Color Compensation (article no. 50503), which is provided by MIKROGEN.

CFX96 (Bio-Rad)

	MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour	Green	Yellow	Orange	Red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N
Mode	all channels			

The data acquisition and evaluation at CFX96 is carried out using the method *calc / data acquisition mode: all channels*. A dye calibration of the CFX96 (Bio-Rad) must be carried out in advance for ATTO Rho12 and ATTO 647N, which is provided by MIKROGEN (CFX96 (Bio-Rad) dye calibration set, article no. 50505).

QuantStudio 5 (Applied Biosystems)

	MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour	Green	Yellow	Orange	Red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N
Excitation	X1 / 470 nm	X2 / 520 nm	X4 / 580 nm	X5 / 640 nm
Emission	M1 / 520 nm	M2 / 558 nm	M4 / 623 nm	M5 / 682 nm
Quencher	[none]	[none]	[none]	[none]

Choose under settings 1. Run mode „standard“, 2. Reference dye “none”, 3. Experiment type “custom”.

Mic (bms)

	MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour	Green	Yellow	Orange	Red

MIKROGEN provides you with validated Mic assay-templates for processing. Please use exclusively MIKROGEN Mic assay-templates. The current MIKROGEN Mic-Assay-Templates are freely available for download on the MIKROGEN homepage (www.mikrogen.de).

Rotor-Gene Q (Qiagen)

	MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour	Green	Yellow	Orange	Red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N
Excitation	470 nm	530 nm	585 nm	625 nm
Emission	510 nm	555 nm	610 nm	660 nm
Gain	5	5	5	5

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 cycles 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 Coronavirus Panel, please contact the manufacturer.

9 Results

The data analysis on the LightCycler® 480 II is carried out using the *Abs Quant/2nd Derivative Max* method.

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 that 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 for the Ct value of the IC of a sample compared to the IC of 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. Using a LightCycler® 480 II the analysis can be carried out either using the *Abs Quant/2nd Derivative Max* method (recommended) or the *Abs Quant/Fit Points* method. Additional information and corresponding instructions are available from MIKROGEN upon request.

		MERS-CoV	SARS-CoV-2 SARS-CoV Bat SARS-like CoV	HCoV	Internal control (IC)
Colour		Green	Yellow	Orange	Red
Reporter dye		FAM	HEX	ATTO Rho12	ATTO 647N
Evaluation with	**LightCycler 480 II**	465-510	533-580	533-610	618-660
	cobas z 480	465-510	540-580	540-610	610-670
	CFX96	FAM	HEX	ROX	Cy5
	QuantStudio 5	FAM	VIC	ROX	ATTO 647N
	Mic	FAM	HEX	ROX	Cy5
	Rotor-Gene Q	green	yellow	orange	red

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

Colour	**MERS-CoV**	**SARS-CoV-2 SARS-CoV Bat SARS-like CoV**	**HCoV**	**Internal control (IC)**
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 MERS-CoV, SARS-CoV-2 / SARS-CoV / Bat SARS-like CoV or HCoV 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 Coronavirus Panel	**MERS-CoV** (n=4)	**SARS-CoV-2** (n=126)	**HCoV** (n=17)
Negative	0	3	0
Positive	4	123	17
Sensitivity [%]	**100**	**97.62**	**100**
CI [%] Confidence interval	**51.01 – 100**	**93.23 – 99.19**	**81.57 – 100**

Table 2: Defined negative samples

*ampli*Cube Coronavirus Panel	**MERS-CoV** (n=24)	**SARS-CoV-2** (n=490)	**HCoV** (n=24)
Negative	24	486	24
Positive	0	4	0
Specificity [%]	**100**	**99.18**	**100**
CI [%] Confidence interval	**86.20 – 100**	**97.92 – 99.68**	**86.20 – 100**

11.2 Analytical sensitivity

11.2.1 *Limit of Detection (LoD) in copies/PCR*

The limit of detection (LoD) of the *ampli*Cube Coronavirus Panel was determined using a dilution series of gBlock DNA of known concentration in a LightCycler® 480 II System (Roche). The 95% limit of detection was determined using probit regression analysis with CombiStats™ Version 5.0 software (Council of Europe). The limit of detection was then confirmed with 20 replicates at the LoD.

Table 3a: Limit of detection (LoD) for MERS and HCoV in copies/PCR

	MERS-CoV	HCoV
LoD [copies/PCR] 95% limit of detection	4.14	12.41*
95% CI [copies/PCR] confidence interval	2.75 – 9.43	8.93 – 22.59

* Information refers to HCoV OC43; HCoV 229E, NL63 and HKU1 were also tested. The limits of detection are between 20.54 and 89.48.

Table 3b: Limit of detection (LoD) for SARS-CoV (LoD) in copies/PCR

	SARS-CoV-2	SARS-CoV	Bat SARS-like CoV
LoD [copies/PCR] 95% limit of detection	2.9	6.74	6.35
95% CI [copies/PCR] confidence interval	1.9 – 6.4	4.12 – 14.68	4.14 – 14.43

11.2.2 *Limit of Detection (LoD) SARS-CoV-2 in International Units*
In addition, the LoD for the SARS-CoV-2 target regions was determined with three independent dilution series (7 dilution levels each) using the WHO standard (First WHO International Standard for SARS-CoV-2 RNA (NIBSC code: 20/146)) including nucleic acid extraction (*alpha*Clean Mag RNA/DNA Kit on M96 Biocomma) on a LightCycler® 480 II system (Roche) and subsequent probit regression analysis (CombiStats™ version 6.0 software (Council of Europe)). The detection limit of the *ampli*Cube Coronavirus Panel is given in International Units (IU) per µl WHO standard.

Tabelle 3c: Limit of detection (LoD) for SARS-CoV-2 in IU/µl WHO standard

	SARS-CoV-2
LoD [IU/µl] 95% limit of detection	2.72
95% CI [IU/µl] confidence interval	1.53 – 6.55

11.3 Analytical specificity

The BLAST trial (www.ncbi.nlm.nih.gov/blast/) shows that the selected primers and probes of the *ampli*Cube Coronavirus Panel 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 in order to demonstrate the analytical specificity of the *ampli*Cube Coronavirus Panel.

Bacteria
Bordetella holmesii
Bordetella parapertussis
Bordetella pertussis
Chlamydia pneumoniae
Mycoplasma pneumoniae

Viruses
Adenovirus A
Cytomegalovirus
Enterovirus
Epstein-Barr Virus
Influenza A
Influenza B
Measles virus
Mumps virus
Parainfluenzavirus
Respiratory syncytial virus (RSV) A
Rhinovirus 58 (A)

None of these samples generated a positive signal. The primers and probes used in the *ampli*Cube Coronavirus Panel 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

	MERS-CoV	SARS-CoV-2	SARS-CoV	Bat SARS-like CoV	HCoV
CV [%] (BAL, H_2O)	1.37	2.11	1.62	2.21	0.58
CV [%] (sputum, H_2O)	2.42	2.32	1.54	3.42	1.91
CV [%] (swab, H_2O)	1.51	1.28	1.47	1.50	1.08
CV [%] (stool, H_2O)	1.94	1.33	1.28	1.98	0.85

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 ≤ 3.42 % for all target genes.

12 Literature

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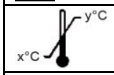
We will be pleased to send you additional literature on request.

13 Explanation of symbols

	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 medical device
LOT	Batch number
REF	Order number
	Use by Expiry date
	Store between x°C and y°C
	Manufacturer

14 Manufacturer and version data

***ampli*Cube Coronavirus Panel**		Article no. **50142**
Instructions for use Valid from		GAACCV003EN 2022-05
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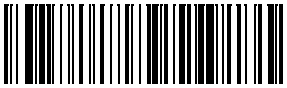
		CE


GAACCV003