MIKROGEN
D I A G N O S T I K

Instruction for Use

*alpha*Cube
respiraRNA 3.0

For the qualitative *in-vitro* detection of the RNA of Influenza A Virus, Influenza B Virus and Respiratory Syncytial Virus (RSV) extracted from biological specimens.

REF	**830508**	**830507**
Σ (32/96)	32	96

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1   Intended Use

The *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit is an assay for the simultaneous detection of RNA of Influenza A Virus, RSV and Influenza B Virus extracted from biological.

2   Pathogen Information

Influenza viruses belong to the family of Orthomyxoviridae and are the causative agent of 'the flu'. Influenza A and B viruses have a single stranded RNA genome, consisting of 8 RNA segments. The genome of Influenza A Viruses is characterized by a high mutation frequency, the so-called ,antigenic drift'. Numerous subtypes of Influenza A Viruses are known. They can be catergorized by their surface antigens H (haemagglutinin) and N (neuraminidase): Influenza A (H1N1) Virus, Influenza A (H5N1) Virus etc. Therefore, yearly in silico analysis of the sequences of newly emerged subtypes is done, to prevent false negative results caused by primer and/or probe mismatches.

Respiratory Syncytial Viruses are enveloped negative-sense, single stranded RNA Viruses of the Paramyxoviridae familiy. RSV is a member of the subfamily Pneumovirinae, genus *Pneumovirus.* RSV are divided into subgroups A and B. RSV is a virus that causes infections of the lungs and respiratory tract. It's so common that most children have been infected with the virus by age 2. RSV can also infect adults.
In adults and older, healthy children, the symptoms of RSV infections are mild and typically mimic the common cold. Self-care measures are usually all that's needed to relieve any discomfort. Infection with RSV can be severe in some cases, especially in premature babies and infants with underlying health conditions. RSV can also become serious in older adults, adults with heart and lung diseases, or anyone with a very weak immune system (immunocompromised).

3   Principle of the Test

The *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit contains specific primers and dual-labeled probes for the amplification of the RNA (cDNA) of Influenza A Virus (M gene, FAM channel), RSV (M gene, ROX channel) and Influenza B Virus (NEP gene, Cy5 channel) extracted from biological specimens.
Furthermore, the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit contains a Control RNA (Internal Process Control, IPC), which is added during RNA extraction and detected in the same reaction by a HEX-labeled probe.
The Control RNA allows the detection of RT-PCR inhibition and acts as control, that the nucleic acid was isolated from the biological specimen.

4   Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of *alpha*Cube respiraRNA 3.0 real time RT-PCR kit.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 442 µl	1 x 1325 µl
Enzyme	blue	1 x 6.4	1 x 19.2 µl
Positive Control	red	1 x 50 µl	1 x 150 µl
Negative Control	green	1 x 50 µl	1 x 150 µl
Control RNA	colourless	1 x 160 µl	1 x 480 µl

5   Equipment and Reagents to be Supplied by User

•   RNA isolation kit (e.g. *alpha*Clean Mag RNA/DNA)
•   PCR grade Water
•   Sterile microtubes
•   Pipets (adjustable volume)
•   Sterile pipet tips with filter
•   Table centrifuge
•   Vortexer
•   Real time PCR instrument
•   Optical PCR reaction tubes with lid or optical reaction plate with optical foil
•   Optional: Liquid handling system for automation

6   Transport, Storage and Stability

*alpha*Cube respiraRNA 3.0 real time RT-PCR is shipped on dry ice or cool packs. All components must be stored at maximum -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.
Up to 20 freeze and thaw cycles are possible.
For convenience, opened reagents can be stored at +2-8°C for up to 6 months.
Protect kit components from direct sunlight during the complete test run.

7   Warnings and Precautions

Read the Instructions for Use carefully before using the product.
Before first use check the product and its components for:

•   Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR procedures.
•   Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
•   Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
•   Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
•   Always wear protective disposable powder-free gloves when handling kit components.

- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.
- Do not combine *alpha*Cube respiraRNA 3.0 real time RT-PCR components of different lot numbers.

8   Sample Material

Starting material for the *alpha*Cube respiraRNA 3.0 real time RT-PCR is RNA isolated from biological specimens (respiratory samples).

9   Sample Preparation

Commercial kits for RNA isolation such as the following are recommended:
- *alpha*Clean Mag RNA/DNA

Please follow the instructions for use of the respective extraction kit.

Important:
In addition to the samples always run a ‚water control' in your extraction. Treat this water control analogous to a sample.
Comparing the amplification of the Control RNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note the chapter ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the manufacturer.

10   Control RNA

A Control RNA is supplied as extraction control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

Add 5 µl Control RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions.

The Control RNA must be added to the Lysis Buffer of the extraction kit.

11   Real time RT-PCR

11.1   Important Points Before Starting:

• Please pay attention to the chapter ‚Warnings and Precautions'.
• Before setting up the real time RT-PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
• The programming of the thermal profile should take place before the RT-PCR set up.
• In every RT-PCR run one Positive Control and one Negative Control should be included.
• Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed (exept the Enzyme) and centrifuged very briefly.
• Due to the high viscosity of the Enzyme (blue lid), prewarming at room temperature for 15 min is recommended.

11.2   Procedure

The Master Mix contains all of the components needed for RT-PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix

Volume per Reaction	Volume Master Mix
13.8 µl Reaction Mix	13.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Real time RT-PCR set up

• Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument/ take an optical PCR reaction plate.
• Pipet **14 µl** of the Master Mix into each optical PCR reaction tube/ the optical PCR reaction plate.
• Add **6 µl** of the eluates from the RNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube/ the optical PCR reaction plate. (Table 3).
• Close the optical PCR reaction tubes/ the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real time PCR

Component	Volume
Master Mix	14.0 µl
Sample	6.0 µl
Total Volume	20.0 µl

11.3   Instrument Settings
For the real time RT-PCR use the thermal profile shown in table 4.

Table 4: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of cDNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Aquisition at the end of this step	60°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to table 5.

Table 5: Overview of the instrument settings required for *alpha*Cube respiraRNA 3.0 real time RT-PCR.

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes		
LightCycler 480II			alphaCube LC480 Color Compensation Kit 2 (831032) required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	Influenza A Virus	465-510	1	10	1
	Control RNA (IPC)	533-580	1	10	2
	RSV	533-610	1	10	2
	Influenza B Virus	618-660	1	10	3
Stratagene Mx3000P/ Mx3005P	Influenza A Virus	FAM	Gain 8		
	Control RNA (IPC)	HEX	Gain 1	Reference Dye: None	
	RSV	ROX	Gain 1		
	Influenza B Virus	Cy5	Gain 4		
AriaMx QuantStudio 5 Bio-Rad CFX96 Bio-Rad CFX Opus 96	Influenza A Virus	FAM			
	Control RNA (IPC)	HEX	Reference Dye: None		
	RSV	ROX			
	Influenza B Virus	Cy5			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Influenza A Virus	Green	Gain 5		
	Control RNA (IPC)	Yellow	Gain 5	Outlier Removal NTC Threshold 15%	
	RSV	Orange	Gain 5		
	Influenza B Virus	Red	Gain 5		
Mic qPCR Cycler	Influenza A Virus	Green	Gain 8		
	Control RNA (IPC)	Yellow	Gain 10		
	RSV	Orange	Gain 10		
	Influenza B Virus	Red	Gain 10		

12   Data Analysis

Following results can occur:

Signal/Ct Values				Interpretation
FAM Channel Influenza A Virus	**Cy5 Channel** Influenza B Virus	**ROX Channel** RSV	**HEX Channel** Control RNA (IPC)	
positive	negative	negative	positive or negative [1]	**Positive result, the sample contains Influenza A Virus RNA.**
negative	**positive**	negative	positive or negative [1]	**Positive result, the sample contains Influenza B Virus RNA.**
negative	negative	**positive**	positive or negative [1]	**Positive result, the sample contains Respiratory Syncytial Virus RNA.**
negative	negative	negative	≤ 34 [2]	**Negative result, the sample contains no Influenza A Virus, no Influenza B Virus and no Respiratory Syncytial Virus RNA.**
negative	negative	negative	negative or > 34 [2]	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.

[1] A strong positive signal in the FAM, Cy5 or ROX channel can inhibit the IPC. In such cases the result for the Control RNA can be neglected.

[2] In case of high C_T values, the IPC should be compared to the water extraction control as described in the chapter 'Assay validation'.

Figure 1 and **Figure 2** show examples for positive and negative real time RT-PCR results.
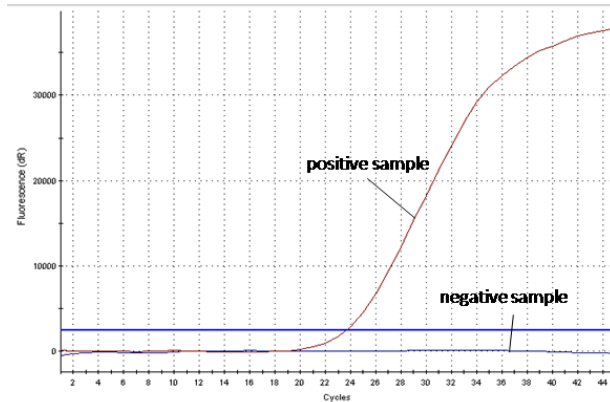

Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.
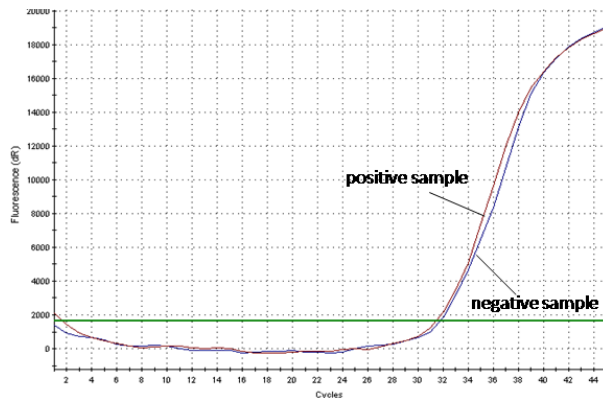

Figure 2: The positive sample and the negative sample show a signal in the Control RNA specific HEX channel (IPC). The amplification signal of the Control RNA in the negative sample shows, that the missing signals in the pathogen specific channels FAM, Cy5 and ROX are not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative sample.

13   Assay Validation

Negative Controls
The Negative Control must show no C_T in the FAM, Cy5, ROX and HEX channel.

Positive Controls
The Positive Control must show a positive (i.e. exponential) amplification curve in the different channels FAM, Cy5 and ROX. The Positive Control must fall below C_T 30.

Internal Controls
The following values for the amplification of the internal control are valid using *alpha*Clean nucleic acid extraction kits *alpha*Clean Mag RNA/DNA. The Control RNA (IPC) must show a positive (i. e. exponential) amplification curve. The Control RNA must fall below a C_T of 34. If the Control RNA is above C_T 34 this points to a purification problem or a strong positive sample that can inhibit the IPC. In the latter case, the assay is valid. It is recommended to perform the extraction of a water control in each run. The IPC in the water control must fall below a C_T of 34. If other nucleic acid extraction kits are used, the customer must define own cutoffs. In this case the C_T value of the Control RNA (IPC) in an eluate from a sample should not be delayed for more than 4 C_T in comparison to an eluate from an extracted water control.

14   Limitations of the Method
- Strict compliance with the Instruction for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR and in vitro diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of RT-PCR inhibitors may cause false negative or invalid results.
- As with any diagnostic test, results of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

15   Troubleshooting
The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR.

No fluorescence signal in the FAM, ROX, Cy5 channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the Influenza A Virus specific amplification, the ROX channel for analysis of the RSV specific amplification and the Cy5 channel for analysis of the Influenza B Virus specific amplification. Select the HEX channel for the amplification of the Control RNA.

Incorrect preparation of the Master Mix	Make sure, the Enzyme is added to the Master Mix (chapter 'Real time RT-PCR').
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with chapter 'Procedure'.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol in chapter 'Instrument Settings'.
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in 'Transport, Storage and Stability'.

Weak or no signal of the Control RNA and simultaneous absence of a signal in the virus specific FAM, ROX or Cy5 channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (chapter 'Real time RT-PCR').
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (chapter 'Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer have been completely removed.
sample material not sufficient	Make sure that enough sample material has been applied to the extraction. Use an appropriate isolation method (chapter 'Sample Preparation') and follow the manufacturer's instructions.
RNA loss during isolation process	In case the Control RNA was added before extraction, the lack of an amplification signal can indicate that the RNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter 'Transport, Storage and Stability'.

Detection of a fluorescence signal in the FAM and/or ROX and/or Cy5 channel of the Negative Control	
Contamination during preparation of the RT-PCR	Repeat the real time RT-PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time RT-PCR.

16   Kit Performance

16.1   Analytical Sensitivity

The limit of detection (LoD) of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit was determined using serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a Stratagene Mx3005P real time PCR instrument.

The LoD of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit for Influenza A Virus is $\leq$ 2.5 genome copies per µl, for Respiratory Syncytial Virus is $\leq$ 0.25 genome copies per µl and for Influenza B Virus $\leq$ 0.125 genome copies per µl.

The sensitivity of the *alpha*Cube respiraRNA 3.0 real time RT-PCR kit was also analysed by testing round robin samples of known status.

16.2   Analytical Specificity

The specificity of the *alpha*Cube respiraRNA 3.0 real time RT-PCR was evaluated with different ring trial samples of known status and different other relevant viruses and bacteria found in clinical samples and basing on in silica analyses.

All ring trial samples were detected correctly. Results are shown in table 6, table 7 and table 8.

The results for the sample analysis are shown in table 9, table 10 and table 11. The results for the in silico analysis are shown in table 12.

Table 6: Ring trial samples tested for the validation of the sensitivity of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit. Results for Influenza A Virus, FAM channel.

Ring trial samples with known status	Ring trial	Expected Result Influenza A Virus **FAM channel**	*alpha*Cube respiraRNA 3.0 Influenza A Virus **FAM channel**
RESPIplus21S-01	QCMD 2021: Respiratory I Plus RESPIplus-21S	negative	negative
RESPIplus21S-02		negative	negative
RESPIplus21S-03		*positive*	*positive*
RESPIplus21S-04		negative	negative
RESPIplus21S-05		negative	negative
RESPIplus21S-06		*positive*	*positive*
RESPIplus21S-07		*positive*	*positive*
RESPIplus21S-08		negative	negative
RESPIplus21S-09		negative	negative
RESPIplus21S-10		negative	negative
432017	Instand: June 2021: Respiratory Viruspanel 2 for Multiplex Tests	*positive*	*positive*
432018		*positive*	*positive*
432019		negative	negative
432020		negative	negative
431017	Instand: June 2021: Respiratory Viruspanel 1 for Multiplex Tests	*positive*	*positive*
431018		negative	negative
431019		negative	negative
431020		negative	negative

Table 7: Ring trial samples tested for the validation of the sensitivity of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit. Results for Influenza B Virus, Cy5 channel.

Ring trial samples with known status	Ring trial	Expected Result Influenza A Virus **Cy5 channel**	*alpha*Cube respiraRNA 3.0 Influenza A Virus **Cy5 channel**
RESPIplus21S-01		negative	negative
RESPIplus21S-02		*positive*	*positive*
RESPIplus21S-03		negative	negative
RESPIplus21S-04		negative	negative
RESPIplus21S-05	QCMD 2021: Respiratory I Plus RESPIplus-21S	negative	negative
RESPIplus21S-06		negative	negative
RESPIplus21S-07		negative	negative
RESPIplus21S-08		negative	negative
RESPIplus21S-09		*positive*	*positive*
RESPIplus21S-10		negative	negative
432017	Instand: June 2021: Respiratory Viruspanel 2 for Multiplex Tests	negative	negative
432018		negative	negative
432019		*positive*	*positive*
432020		negative	negative
431017	Instand: June 2021: Respiratory Viruspanel 1 for Multiplex Tests	negative	negative
431018		*positive*	*positive*
431019		negative	negative
431020		negative	negative

Table 8: Ring trial samples tested for the validation of the sensitivity of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit. Results for RSV, ROX channel.

Ring trial samples with known status	Ring trial	Expected Result RSV **ROX channel**	*alpha*Cube respiraRNA 3.0 RSV **ROX channel**
RESPIplus21S-01		negative	negative
RESPIplus21S-02		negative	negative
RESPIplus21S-03		negative	negative
RESPIplus21S-04		*positive*	*positive*
RESPIplus21S-05	QCMD 2021: Respiratory I Plus RESPIplus-21S	negative	negative
RESPIplus21S-06		negative	negative
RESPIplus21S-07		negative	negative
RESPIplus21S-08		negative	negative
RESPIplus21S-09		negative	negative
RESPIplus21S-10		*positive*	*positive*
432017	Instand: June 2021: Respiratory Viruspanel 2 for Multiplex Tests	*positive*	*positive*
432018		negative	negative
432019		*positive*	*positive*
432020		negative	negative
431017	Instand: June 2021: Respiratory Viruspanel 1 for Multiplex Tests	*positive*	*positive*
431018		negative	negative
431019		negative	negative
431020		negative	negative

Table 9: Eluted RNA/DNA from bacterial and viral pathogens tested for the determination of the analytical specificity of *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit, FAM channel (Influenza A Virus).

sample	Expected Result Influenza A Virus **FAM channel**	*alpha*Cube respiraRNA 3.0 Influenza A Virus **FAM channel**
Adenovirus C2	negative	negative
Adenovirus 41	negative	negative
Bordetella parapertussis	negative	negative
Bordetella pertussis	negative	negative
Cytomegalievirus	negative	negative
Enterovirus Coxsackie B3	negative	negative
Emterovirus D68	negative	negative
Epstein-Barr Virus	negative	negative
Coronavirus OC43	negative	negative
Coronavirus NL63	negative	negative
Human Herpesvirus 6	negative	negative
Influenza Virus A H1N1	*positive*	*positive*
Influenza Virus A H3N2	*positive*	*positive*
Influenza Virus A H5N1	*positive*	*positive*
Influenza Virus B	negative	negative
Human Coronavirus MERS-CoV	negative	negative
Metapneumovirus Type A1	negative	negative
Metapneumovirus Type A2	negative	negative
Staphylococcus aureus (MRSA)	negative	negative
Mycobacterium tuberculosis complex	negative	negative
Mycoplasma pneumoniae	negative	negative
Parainfluenza Type 1	negative	negative
Parainfluenza Type 2	negative	negative
Parainfluenza Type 3	negative	negative
Parainfluenza Type 4	negative	negative
Parechovirus 3	negative	negative
Pneumocystis jirovecii	negative	negative
Respiratory Syncytial Virus A	negative	negative
Respiratory Syncytial Virus B	negative	negative
Rhinovirus Type 5	negative	negative
Rotavirus G1 [P8]	negative	negative
SARS-CoV-2	negative	negative

Table 10: Eluted RNA/DNA from bacterial and viral pathogens tested for the determination of the analytical specificity of *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit, Cy5 channel (Influenza B Virus).

sample	Expected Result Influenza B Virus **Cy5 channel**	*alpha*Cube respiraRNA 3.0 Influenza B Virus **Cy5 channel**
Adenovirus C2	negative	negative
Adenovirus 41	negative	negative
Bordetella parapertussis	negative	negative
Bordetella pertussis	negative	negative
Cytomegalievirus	negative	negative
Enterovirus Coxsackie B3	negative	negative
Emterovirus D68	negative	negative
Epstein-Barr Virus	negative	negative
Coronavirus OC43	negative	negative
Coronavirus NL63	negative	negative
Human Herpesvirus 6	negative	negative
Influenza Virus A H1N1	negative	negative
Influenza Virus A H3N2	negative	negative
Influenza Virus A H5N1	negative	negative
Influenza Virus B	*positive*	*positive*
Human Coronavirus MERS-CoV	negative	negative
Metapneumovirus Type A1	negative	negative
Metapneumovirus Type A2	negative	negative
Staphylococcus aureus (MRSA)	negative	negative
Mycobacterium tuberculosis complex	negative	negative
Mycoplasma pneumoniae	negative	negative
Parainfluenza Type 1	negative	negative
Parainfluenza Type 2	negative	negative
Parainfluenza Type 3	negative	negative
Parainfluenza Type 4	negative	negative
Parechovirus 3	negative	negative
Pneumocystis jirovecii	negative	negative
Respiratory Syncytial Virus A	negative	negative
Respiratory Syncytial Virus B	negative	negative
Rhinovirus Type 5	negative	negative
Rotavirus G1 [P8]	negative	negative
SARS-CoV-2	negative	negative

Table 11: Eluted RNA/DNA from bacterial and viral pathogens tested for the determination of the analytical specificity of *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit, ROX channel (Respiratory Syncytial Virus).

sample	Expected Result RSV **ROX channel**	*alpha*Cube respiraRNA 3.0 RSV **ROX channel**
Adenovirus C2	negative	negative
Adenovirus 41	negative	negative
Bordetella parapertussis	negative	negative
Bordetella pertussis	negative	negative
Cytomegalievirus	negative	negative
Enterovirus Coxsackie B3	negative	negative
Emterovirus D68	negative	negative
Epstein-Barr Virus	negative	negative
Coronavirus OC43	negative	negative
Coronavirus NL63	negative	negative
Human Herpesvirus 6	negative	negative
Influenza Virus A H1N1	negative	negative
Influenza Virus A H3N2	negative	negative
Influenza Virus A H5N1	negative	negative
Influenza Virus B	negative	negative
Human Coronavirus MERS-CoV	negative	negative
Metapneumovirus Type A1	negative	negative
Metapneumovirus Type A2	negative	negative
Staphylococcus aureus (MRSA)	negative	negative
Mycobacterium tuberculosis complex	negative	negative
Mycoplasma pneumoniae	negative	negative
Parainfluenza Type 1	negative	negative
Parainfluenza Type 2	negative	negative
Parainfluenza Type 3	negative	negative
Parainfluenza Type 4	negative	negative
Parechovirus 3	negative	negative
Pneumocystis jirovecii	negative	negative
Respiratory Syncytial Virus A	*positive*	*positive*
Respiratory Syncytial Virus B	*positive*	*positive*
Rhinovirus Type 5	negative	negative
Rotavirus G1 [P8]	negative	negative
SARS-CoV-2	negative	negative

Table 12: Inclusivity of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit Primers and Probes (in silico analysis).

12 - 5000 whole genome sequences			Homology	Comment
NCBI / GISAID	RSV	Forward Primer	12 sequences: 100%	no mismatch
		Reverse Primer	2 sequences: 100%	10 sequences: 96% (1 mismatch)
		Probe	10 sequences: 100%	2 sequences: 96% (1 mismatch)
NCBI / GISAID	Flu B	Forward Primer	1000 sequences: 100%	no mismatch
		Reverse Primer	1000 sequences: 100%	no mismatch
		Probe	998 sequences: 100%	2 sequences: 96% (1 mismatch)
NCBI / GISAID	Flu A	Forward Primer	5000 sequences: 100%	no mismatch
		Reverse Primer	5000 sequences: 100%	no mismatch
		Probe	5000 sequences: 100%	no mismatch

16.3 Linear Range

The linear range of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts of the target sequences with both thermal profiles.

Figure 3: Determination of the linear range of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit for Influenza A Virus in the FAM channel
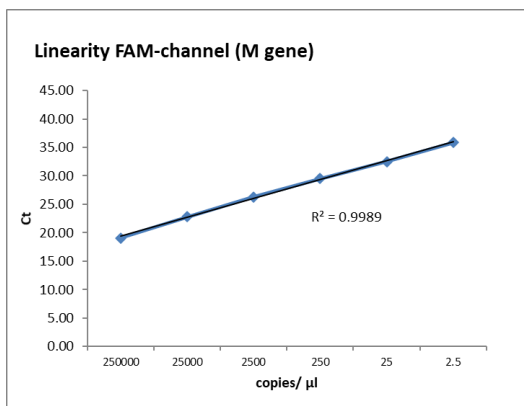
Figure 4: Determination of the linear range of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit for Influenza B Virus in the Cy5 channel
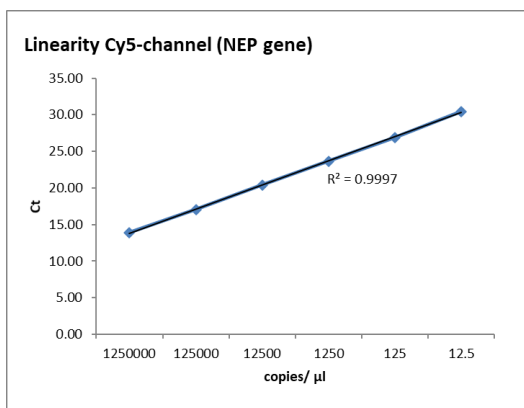
Figure 5: Determination of the linear range of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit for Respiratory Syncytial Virus in the ROX channel
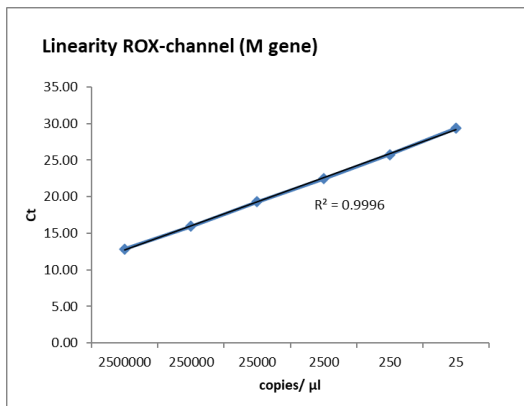
16.4 Precision

The precision of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit was determined as intra-assay variability, inter-assay variability and inter-lot variability.

Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of an Influenza A Virus in vitro tanscript, an Influenza B Virus in vitro transcript, a Respiratory Syncytial Virus in vitro transcript and on the threshold cycle of the Control RNA (IPC). The results are shown in table 13.

Table 13: Precision of the *alpha*Cube respiraRNA 3.0 real time RT-PCR Kit
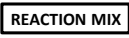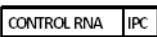
Influenza A Virus (FAM)	copies/ µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.24	0.74
Inter-Assay-Variability	25	0.78	2.36
Inter-Lot-Variability	25	0.67	2.03
Influenza B Virus (Cy5)	copies/ µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	12.5	0.32	1.05
Inter-Assay-Variability	12.5	0.08	0.26
Inter-Lot-Variability	12.5	0.13	0.44
Respiratory Syncytial Virus (ROX)	copies/ µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	2.5	0.28	0.85
Inter-Assay-Variability	2.5	0.23	0.71
Inter-Lot-Variability	2.5	0.17	0.53
IPC (HEX)	copies/ µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	250	0.49	1.86
Inter-Assay-Variability	250	0.59	2.19
Inter-Lot-Variability	250	0.59	2.23

16.5 Diagnostic Sensitivity

The diagnostic sensitivity of real time (RT-)PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various biological specimens. DNA/RNA extraction reagents are not part of the *alpha*Cube real time (RT-)PCR kits. *alpha*Cube real time (RT-)PCR kits include an extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-)PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.

Therefore, gerbion guarantees the analytical sensitivities and specificities of the real time (RT-)PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. gerbion does not guarantee diagnostic sensitivities. If diagnostic sensitivities are mentioned in manuals of *alpha*Cube real time (RT-)PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods. It is the responsibility of the user to qualify the extraction methods used for DNA/RNA isolation from biological samples.

17 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid	REF	Catalog number
RNA	Ribonucleid Acid	$\boxed{\Sigma}$	Contains sufficient for <n> test
PCR	Polymerase Chain Reaction	-18°C	Upper limit of temperature
RT	Reverse Transcription		Manufacturer
RSV	Respiratory Syncytial Virus		Use by YYYY-MM-DD
REACTION MIX	Reaction Mix	LOT	Batch code
ENZYME	Enzyme	CONT	Content
CONTROL +	Positive Control		Consult instructions for use
CONTROL −	Negative Control	IVD	*In vitro* diagnostic medical device
CONTROL RNA IPC	Control RNA (IPC)		

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18 Literature

[1]   Lothar Thomas, Labor und Diagnose: Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik, 8. Auflage, 2012, TH-Books, ISBN-10: 3980521583