MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube Entero 2.0

For qualitative *in-vitro* detection of Enterovirus RNA (Enterovirus, Coxsackievirus, Echovirus, Poliovirus) in clinical specimens and environmental samples.

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1 Intended Use

The *alpha*Cube Entero 2.0 is a real time RT-PCR assay for the detection of Enterovirus RNA (Enterovirus, Coxsackievirus A and B, Echovirus, Poliovirus type 1-3) in clinical specimens and environmental samples.

2 Pathogen Information

Enteroviruses are highly contagious pathogens belonging to the family of Picornaviridae. They are small, non-enveloped RNA-viruses which are very resistant to environmental conditions. Even at pH 3-9 or in the presence of detergences Enteroviruses remain infectious. The transmission from person to person happens mainly fecal-orally. Contaminated foods and drinking water are important sources of infection. The viruses can be egested in stool even weeks after an acute infection.

Infections with Enteroviruses can occur throughout the year, however, in summer, contaminated water in swimming pools or lakes lead to increases in the number of Enterovirus infections.

The symptoms caused by Enteroviruses are numerous: infections of the upper respiratory tract, undifferentiated fever, herpangina, hand-foot-mouth-disease, rash disease, paralyses, etc.

3 Principle of the Test

*alpha*Cube Entero 2.0 contains specific primers and dual-labeled probes for the amplification and detection of Enterovirus RNA in clinical specimens and environmental samples.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM channel.

Furthermore, *alpha*Cube Entero 2.0 contains a Control RNA, which is added during RNA extraction and detected in the same reaction by a differently labeled probe.

The Control RNA allows the detection of RT-PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimens and environmental samples.

The fluorescence of the Control RNA is measured in the VIC®/HEX/JOE/TET channel.

4 Package Contents

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

Table 1: Components of *alpha*Cube Entero 2.0

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
Enzyme	blue	1 x 6.4 µl	1 x 19.2 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control RNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. *alpha*Clean Pure RNA/DNA or *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
- Optional: Liquid handling system for automation
- Optional: VLP-RNA (Virus-Like Particles, please look at page 5 for details)

6 Transport, Storage and Stability

*alpha*Cube Entero 2.0 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 Important Notes

- *alphaCube* Entero 2.0 must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the RT-PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine *alpha*Cube Entero 2.0 components of different lot numbers.

9 Sample Material

Starting material for the *alpha*Cube Entero 2.0 real time RT-PCR is RNA isolated or released from clinical specimens and environmental samples.

10 Sample Preparation

*alpha*Cube Entero 2.0 is suitable for the detection of Enterovirus RNA isolated from clinical specimens and environmental samples with appropriate isolation methods.
Commercial kits for RNA isolation such as the following are recommended:

- *alpha*Clean Pure RNA/DNA
- *alpha*Clean Mag RNA/DNA

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 11 ‚Control RNA'.

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

11 Control RNA

A Control RNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.
The Virus-Like Particles (VLP-RNA) is not supplied, but can be used for patient-side extraction by spiking of VLP-RNA directly to the sample.

RNA isolation from clinical specimens and environmental samples
a) Control RNA or VLP-RNA used as Extraction Control:
*alpha*Cube Entero 2.0 Control RNA or VLP-RNA is added to the RNA extraction.
Add 5 µl Control RNA or VLP-RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions. Please follow protocol A.

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

b) Control RNA used as Internal Control of the real time RT-PCR:
If only inhibition will be checked please follow protocol B.

12 Real time RT-PCR

12.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Important Notes'.
- 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 PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents - except the Enzyme - should be thawed completely at room temperature, thoroughly mixed (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

12.2 Procedure

If the Control RNA or VLP-RNA is used to control both, the real time RT-PCR and the RNA isolation procedure, please follow protocol A. If the Control RNA is solely used to detect possible inhibition of the real time RT-PCR, please follow protocol B.

Protocol A

The Control RNA or VLP-RNA was added during RNA extraction (see chapter 11 ‚Control RNA'). In this case, prepare the Master Mix according to Table 2.

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 (Control RNA was added during RNA extraction)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Protocol B

The Control RNA is used for the control of the real time RT-PCR only (see chapter 11 ‚Control RNA'). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real 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.

Important: Dilute the Control RNA **1:10** in PCR grade Water (e.g. 1 µl Control RNA + 9 µl PCR grade Water) before adding it to the Master Mix.

Table 3: Preparation of the Master Mix (Control RNA is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)
0.2 µl Control RNA* (diluted 1:10)	0.2 µl x (N+1)*

*The increase in volume caused by adding the Control RNA is not taken into account when preparing the PCR assay.

Protocol A and B: 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.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 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 (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time RT-PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time RT-PCR use the thermal profile shown in table 5.

Table 5: real time RT-PCR thermal profile

Discription	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	45°C	1
Initial Denaturation	2 min	95°C	1
Amplification			
Denaturation	5 sec	95°C	45
Annealing	20 sec Aquisition at the end of this step	55°C	
Extension	10 sec	72°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to table 6.

Table 6: Overview of the instrument settings required for *alpha*Cube Entero 2.0.

Real time PCR Instrument	Parameter	Detection Channel	Notes	
LightCycler 480I	Enteroviruses	483-533	pre-installed universal CC FAM (510) – VIC (580)	
	Control RNA	523-568		
LightCycler 480II	Enteroviruses	465-510		
	Control RNA	533-580		
Stratagene Mx3000P / Mx3005P	Enteroviruses	FAM	Gain 8	Reference Dye: None
	Control RNA	HEX	Gain 1	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Enteroviruses	Green	Gain 5	
	Control RNA	Yellow	Gain 5	

13 Data Analysis

The virus specific amplification is measured in the FAM channel. The amplification of the Control RNA or VLP-RNA is measured in the VIC®/HEX/JOE/TET channel.

Following results can occur:

- **A signal in the FAM channel is detected:**
  The result is positive, the sample contains Enterovirus RNA.
  In this case, detection of a signal of the Control RNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the Control RNA.
- **No signal in the FAM channel, but a signal in the VIC®/HEX/JOE/TET channel is detected:**
  The result is negative, the sample does not contain Enterovirus RNA.
  The signal of the Control RNA excludes the possibilities of RNA isolation failure (in case the Control RNA is being used as an Extraction Control) and/or real time RT-PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“).
- **Neither in the FAM nor in the VIC®/HEX/JOE/TET channel a signal is detected:**
  A diagnostic statement cannot be made.
  The RNA isolation was not successful or an inhibition of the RT-PCR has occurred. In case the Control RNA was added during RNA isolation and not directly to the PCR Master Mix, the Negative Control is negative in both channels.

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

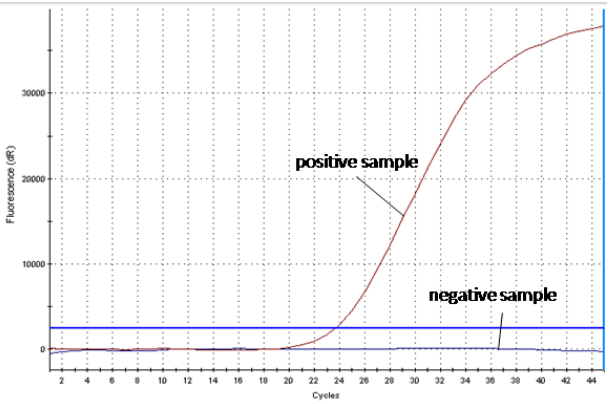

Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

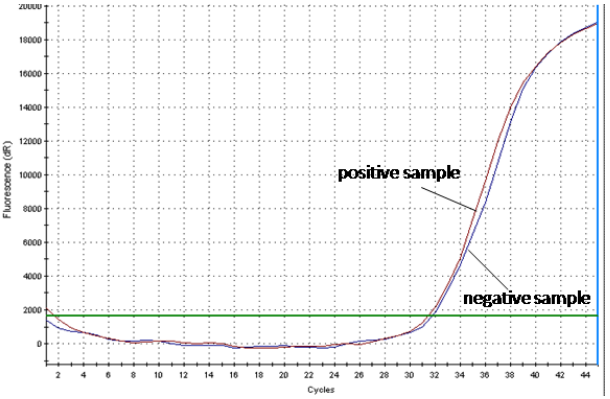

Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA specific VIC®/HEX/JOE/TET channel. The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus specific FAM channel is not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative.

14 Assay Validation

Set a threshold as follows:

Negative Controls
All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls
All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls
All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 33.

15 Limitations of the Method

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude an Enterovirus infection.

16 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 channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the virus specific amplification and the VIC®/HEX/JOE/TET channel for the amplification of the Control RNA.
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with ‚Procedure' on page 6.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 8).
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', page 4.

Weak or no signal of the Control RNA and simultaneous absence of a signal in the virus specific FAM channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 6).
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. An additional centrifugation step at high speed is recommended before elution of the RNA.
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 ‚Transport, Storage and Stability', page 4.
Detection of a fluorescence signal in the FAM 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 occured 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.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the *alpha*Cube Entero 2.0 65 positive and 30 negative clinical samples, previously characterized by virus isolation in cell cultures, were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 %.
The positive predictive value was found to be 100 %, the negative predictive value showed to be 100 %.

Table 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	**negative samples**
*alpha*Cube Entero 2.0 positive	65	0
*alpha*Cube Entero 2.0 negative	0	30
Sensitivity	100 %	
Specificity	100 %	

17.2 Analytical Sensitivity

The limit of detection (LoD) of *alpha*Cube Entero 2.0 was determined using serial dilutions of Enterovirus cell culture supernatants in culture medium in a Stratagene Mx3000 real time PCR instrument. Total nucleic acids were extracted using *alpha*Clean Pure RNA/DNA according to the manufacturer´s instructions. Each sample (200 µl of diluted supernatant) was supplemented with 5 µl Control-RNA prior to extraction. Total nucleic acids were eluted with 50 µl and 4 µl of the eluates were applied to the subsequent real time RT-PCR.
The LoD of *alpha*Cube Entero 2.0 for *Enteroviruses* is $\leq$0.2 TCID 50 per reaction each.

Table 8: Strains tested for the validation of the sensitivity of *alpha*Cube Entero 2.0.

Strain	**TCID 50 / ml**	**Dilution/LoD**	**corresponding TCID 50**
Coxsackievirus A9	1.25×10^{7}	1×10^{-6}	0.2
Coxsackievirus A16	1×10^{6}	1×10^{-6}	0.016
Coxsackievirus B3	1×10^{8}	1×10^{-7}	0.16
Enterovirus 68	3.2×10^{5}	2×10^{-5}	0.05
Echovirus 30	1×10^{6}	1×10^{-5}	0.16

17.3 Analytical Specificity

The specificity of *alpha*Cube Entero 2.0 was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The *alpha*Cube Entero 2.0 real time RT-PCR showed a positive result for the samples containing *Enteroviruses,* whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 9.
Furthermore QCMD and INSTAND ring trials from 2010 – 2015 has been passed successfully with a score of 100% correct results each.

Table 9: Bacterial and viral pathogens tested for the determination of the analytical specificity of *alpha*Cube Entero 2.0.

Pathogen	*alpha*Cube Entero 2.0
Coxsackievirus A7	positive
Coxsackievirus A9	positive
Coxsackievirus A16	positive
Coxsackievirus A24	positive
Coxsackievirus B3	positive
Coxsackievirus B4	positive
Coxsackievirus B5	positive
Coxsackievirus B6	positive
Echovirus 9	positive
Echovirus 11	positive
Echovirus 20	positive
Echovirus 30	positive
Enterovirus 71	positive
Enterovirus 68	positive
Poliovirus 1	positive
Poliovirus 2	positive
Poliovirus 3	positive
Influenza A Virus	negative
Parechovirus 3	negative
Norovirus	negative
Rotavirus	negative
Adenovirus	negative
Salmonella thyphimurium	negative
Citrobacter freundii	negative
Yersinia enterocolitica	negative
Listeria monocytogenes	negative
Shigella boydii	negative
Shigella sonnei	negative
Shigella flexneri	negative
E. coli	negative

18 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid
RNA	Ribonucleid Acid
PCR	Polymerase Chain Reaction
RT	Reverse Transcription
LoD	Limit of Detection
TCID 50	Tissue Culture Infective Dose 50%
REACTION MIX	Reaction Mix
ENZYME	Enzyme
CONTROL +	Positive Control
CONTROL −	Negative Control
CONTROL RNA IC	Control RNA

REF	Catalog number
Σ	Contains sufficient for <n> test
-18°C	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM-DD
LOT	Batch code
CONT	Content
	Consult Instructions for Use
IVD	*In vitro* diagnostic medical device
CE	European Conformity

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19 Literature

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