Instruction Manual

virellaEntero 2.0 LC

real time RT-PCR Kit

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

REF	G01096-32	G01096-96
Σ	32	96

- 18°C

gerbion gmbH & Co. KG
Remsstr. 1
70806 Kornwestheim
Germany
phone: +49 7154 806 20 0
fax: +49 7154 806 20 29
e-mail: info@gerbion.com
www.gerbion.com

CE

Inhaltsverzeichnis

1 Components

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

Table 1: Components of the virellaEntero 2.0 LC real time RT-PCR Kit.

	Label	Lid Colour	Content	
			32	96
K1	Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
K2	Enzyme	blue	1 x 6,4 µl	1 x 19,2 µl
K3	Positive Control	red	1 x 50 µl	1 x 100 µl
K4	Negative Control	green	1 x 50 µl	1 x 100 µl
K5	Control RNA	red	1 x 160 µl	2 x 240 µl

2 Abbreviations and Symbols

RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
RT	Reverse Transcription
cDNA	complementary Deoxyribonucleic Acid
	Catalog number
	Contains sufficient for <n> test
	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM
	Consult instructions for use
	Batch code
	Positive Control

CONTROL —	Negative Control
REACTION MIX	Reaction Mix
CONTROL RNA IC	Control RNA

3 Transport and Storage

The **virellaEntero 2.0 LC real time RT-PCR Kit** is shipped on dry ice. All components must be stored -18°C in the dark immediately after receipt.
Do not use reagents after the date of expiry printed on the package. After initial usage, reagents are stable for up to six months.

4 Intended Use

The **virellaEntero 2.0 LC real time RT-PCR Kit** is an assay for the detection of Enterovirus RNA (Enterovirus, Coxsackievirus A and B, Echovirus, Poliovirus type 1-3) in clinical specimens and environmental samples using the capillary system of the LightCycler®1.5 and 2.0.

5 Sample Material

Starting material for the **virellaEntero 2.0 LC real time RT-PCR Kit** is Enterovirus RNA isolated from clinical specimens and environmental samples.

6 Quality Control

In accordance with gerbion's ISO-certified Quality Management System, each lot of the **virellaEntero 2.0 LC real time RT-PCR Kit** is tested against predetermined specifications to ensure consistent product quality.

7 Product Warranty

gerbion guarantees the performance of all products when used according to the instructions given in the Instruction Manual. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, gerbion will replace it free of charge or refund the price. We reserve the right to change, alter, or modify any product to enhance its performance and design.

8 Introduction

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.

9 Principle of the Test

The **virellaEntero 2.0 LC real time RT-PCR Kit** contains specific primers and hydrolysis probes for the detection of Enterovirus RNA (Enterovirus, Coxsackievirus, Echovirus, Poliovirus) in clinical specimens and environmental samples after the extraction of RNA from the sample material. The reverse transcription (RT) of viral RNA to cDNA and the subsequent amplification of virus specific fragments are performed in a one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the Polymerase. The emitted fluorescence is measured in the 530 nm (F1 channel of the LightCycler®).

Furthermore, the **virellaEntero 2.0 LC real time RT-PCR Kit** real time PCR Kit contains a Control RNA (K5), which is detected in a second amplification system. Added during RNA extraction, the Control RNA (K5) allows not only for the detection of RT-PCR inhibition but also detects possible mistakes during RNA extraction. This greatly reduces the risk of false-negative results. The amplification of the Control RNA (K5) can be measured either in the F2- or in the F3- channel (LightCycler® 1.5) or in the F4- or F6- channel (LightCycler® 2.0).

10 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. **NukEx Pure** RNA/DNA, gerbion Cat. No. G05004)
- 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

11 Important Notes

- The **virellaEntero 2.0 LC real time RT-PCR Kit** 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.

12 General Precautions

- Stick to the protocol described in the Instruction Manual.
- 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.
- The Enzyme (K2) is liquid even at -18°C. Take it out of the freezer shortly before usage and put it back immediately.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanohol-free decontaminant.
- Do not combine **virellaEntero 2.0 LC real time RT-PCR Kit** components of different lot numbers.

13 Isolation of Viral RNA

The **virellaEntero 2.0 LC real time RT-PCR Kit** is suitable for the detection of Enterovirus RNA (Enterovirus, Coxsackievirus, Echovirus, Poliovirus) extracted from clinical specimens and environmental samples with suitable isolation methods.

Commercial kits for RNA isolation such as the following are recommended:

- **NukEx Pure RNA/DNA**, gerbion Cat. No. G05004

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 (K5) 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' on page 8.

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

Further information about RNA isolation is to be found in the extraction kit manual or from the extraction kit manufacturer‘s technical service.

14 Control RNA (K5)

A Control RNA (K5) is supplied to be used as Extraction Control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

Control RNA (K5) used as Extraction Control:

virellaEntero 2.0 LC real time RT-PCR Kit Control RNA (K5) is added prior to the RNA extraction.

To this end, multiply the buffer volume needed per extraction with the number of samples (including at least one water control) (N) plus 1 to compensate for inaccuracies in pipetting (N+1). Add 5 µl Control RNA (K5) per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer‘s instructions.

If the extraction protocol includes an incubation step of the sample in the first buffer, the Control RNA (K5) is to be added to each sample individually after incubation.

The Control RNA (K5) must not be added to the sample material directly.

Control RNA (K5) used as Internal Control of the real time RT-PCR:

If control of the RNA extraction is not desired, the Control RNA (K5) can be used as Internal Control of the real time RT-PCR only. To that end, the Control RNA (K5) is to be added directly to the real time RT-PCR Master Mix. Set up the real time RT-PCR according to protocol B.

15 Real time RT-PCR

15.1 Important Points Before Starting::

- Please pay attention to the ‚Important Notes‘ on page 6.
- 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 PCR set up.

- In every PCR run at least one Positive Control (K3) and one Negative Control (K4) should be included.
- Before each use, all reagents - except the Enzyme (K2) - should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the Reaction Mix (K1) but mix by pipetting up and down repeatedly), and centrifuged very briefly. Then place all reagents on ice or on a cooling block (+2 to +8°C).

15.2 Procedure

If the Control RNA (K5) is used to control both the real time RT-PCR and the RNA isolation procedure, please follow protocol A. If the Control RNA (K5) is solely used to detect possible inhibition/failure of the real time RT-PCR, please follow protocol B.

Protocol A

The Control RNA (K5) was added during RNA extraction (see ‚Control RNA', page 8). In this case, prepare the Master Mix on ice or in a cooling block (+2 to +8°C) 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 (K5) was added during RNA extraction)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix (K1)	15.8 µl x (N+1)
0.0 µl Control RNA (K5)	0.0 µl x (N+1)
0.2 µl Enzyme (K2)	0.2 µl x (N+1)

Protocol B

The Control RNA (K5) is used for the control of the real time RT-PCR only (see ‚Control RNA', page 8). In this case, prepare the Master Mix on ice or in a cooling block (+2 to +8°C) 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 (K5) **1:100** in sterile dH_2O (e.g. 1 µl Control RNA (K5) + 99 µl dH_2O) before adding it to the Master Mix.

Table 3: Preparation of the Master Mix (Control RNA (K5) is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix (K1)	15.8 µl x (N+1)
0.2 µl Control RNA (K5)* **(diluted 1:100)**	0.2 µl x (N+1)*
0.2 µl Enzyme (K2)	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. The sensitivity of the detection system is not impaired.

Protocol A and B: real time RT-PCR set up

- Put the number of optical PCR reaction tubes needed into the cooling block.
- 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 (K3), and the Negative Control (K4) 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 PCR

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

16 Instrument Settings

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

Table 5: real time RT-PCR thermal profile

Step	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	45 °C	1
Initial Denaturation	2 min	95°C	1
Amplification of cDNA			
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 the **virellaEntero 2.0 LC** real time PCR.

Real time RT-PCR Insttrument	Parameter	Detection	Channel Notes
LightCycler 480I	Entero	483-533	Color Compensation Kit required
	Control RNA	523-568	
LightCycler 480II	Entero	465-510	
	Control RNA	498-580	
Stratagene Mx3000P / Mx3005P	Entero	FAM	Gain 8 Reference Dye: None
	Control RNA	HEX	Gain 1
ABI 7500	Entero	FAM	Option Reference Dye ROX: NO
	Control RNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Entero	Green	
	Control RNA	Yellow	

17 Data Analysis

The virus specific amplification is measured in the 530 nm channel (LightCycler® F1). The amplification of the Control RNA (K5) can be measured either in the 640 nm or in the 705 nm channel (LightCycler® 1.5: F2 or F3; LightCycler® 2.0: F4 or F6).

Following results can occur:

- A signal in the F1 channel is detected:
  The result is positive, the sample contains Enterovirus RNA.

  In this case, detection of a signal of the Control RNA (K5) in the F2 and F3 channel (LightCycler® 1.5) respectively in the F4 and F6 channel (LightCycler® 2.0) is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the Control RNA (K5).

- No signal in the F1 channel, but a signal in the F2 and F3 channel (LightCycler® 1.5) respectively in the F4 and F6 channel (LightCycler® 2.0) is detected:
  The result is negative, the sample does not contain Enterovirus RNA.

  The signal of the Control RNA (K5) excludes the possibilities of RNA isolation failure (in case the Control RNA (K5) 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“, page15).

- Neither in the F1 nor in the F2 and F3 channel (LightCycler® 1.5) respectively in the F4 and F6 channel (LightCycler® 2.0) 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 (K5) was added during RNA isolation and not directly to the PCR Master Mix, the Negative Control (K4) is negative in both channels.

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

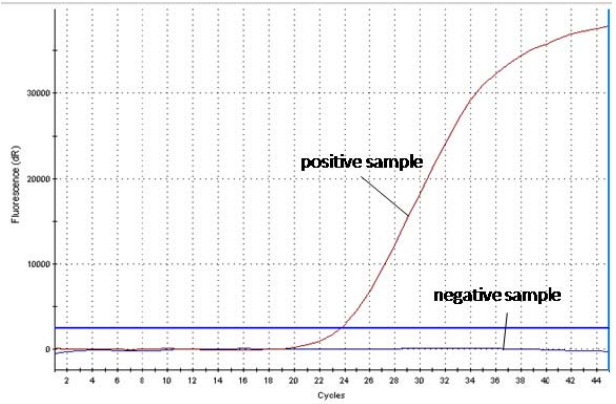

Figure 1: The positive sample shows virus-specific amplification in the F1 channel, whereas no fluorescence signal is detected in the negative sample.

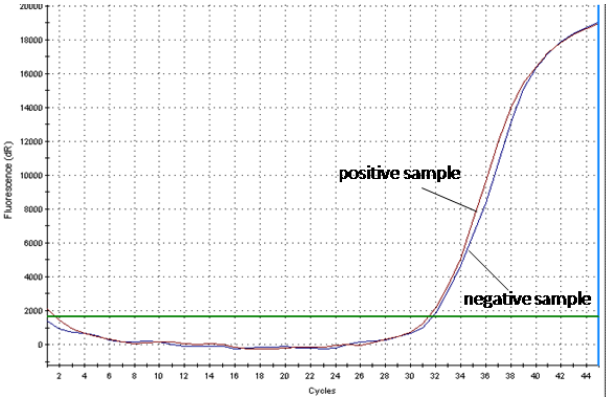

Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA-specific F2 and F3 channel (LightCycler® 1.5) respectively in the F4 and F6 channel (LightCycler® 2.0). The amplification signal of the Control RNA (K5) in the negative sample shows, that the missing signal 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.

18 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the F1 channel the Positive Control (K3)	
The selected channel for analysis does not comply with the protocol	Select the F1 channel for analysis of the virus specific amplification and either the 640 nm or the 705 nm channel (LightCycler® 1.5: F2 or F3; LightCycler® 2.0: F4 or F6) for the amplification of the Control RNA (K5).
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with ‚Procedure' on page 9.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 11).
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 and Storage', page 4.
Weak or no signal of the Control RNA (K5) and simultaneous absence of a signal in the virus specific F1 channel	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 9).
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Isolation of Viral RNA', page 7) and follow the manufacturer's instructions. Make sure that the ethanol-containing wash buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the RNA.
RNA loss during isolation	In case the Control RNA (K5) was added before

process	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 and Storage', page 4.
Detection of a fluorescence signal in the F1 channel of the Negative Control (K4)	
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 (K3) 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.

19 Other Products

A number of products related to real time PCR and nucleic acid isolation is available from gerbion GmbH & Co. KG. More information as well as the complete Product Catalogue is available on www.gerbion.com.

Product	Description	Cat. No.
NukEx Pure RNA/DNA	Column based kit for high yield isolation of nucleic acids from a wide variety of sample material. For 50 and 200 reactions.	G05004-50 G05004-200
NukEx Collection Tubes	500 NukEx Collection Tubes for use with NukEx Spin Columns.	G06008
NukEx Pestle 1.5 ml	100 disposable PBTP pestles for use in 1.5 ml reaction tubes. Individually packed. DNase-free, RNase-free, non-pyrogenic.	G06006
NukEx TS	Shredding material aliquoted in 1.5 or 2.0 ml safe lock tubes or 2.0 ml screw cap tubes for the manual preparation of samples such as tissue or insects.	G06007-1.5 G06007-2.0 G06007-2.0 sc
Proteinase K	Proteinase K. Molecular Biology Grade. 50 mg.	G07001
DNase I	DNase I, RNase-free is an endonuclease that digests single- and double-stranded DNA. It hydrolyzes phosphodiester bonds producing mono- and oligodeoxyribo-nucleotides with 5'-phosphate and 3'-OH groups. 10,000 U in solution (10 U/µl) supplied with 10 x DNase I Reaction Buffer.	G07002