

Instruction for Use

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 using LightCycler[®] 1.5 / 2.0.

REF

G01096-32

G01096-96



32

96



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



Index

1	Intended Use	3
2	Pathogen Information	3
3	Principle of the Test	3
4	Package Contents	4
5	Equipment and Reagents to be Supplied by User	4
6	Transport, Storage and Stability	4
7	Important Notes	5
8	General Precautions	5
9	Sample Material	5
10	Sample Preparation	5
11	Control RNA	6
12	Real time RT-PCR	6
12.1	Important Points before Starting:	6
12.2	Procedure	7
12.3	Instrument Settings	9
13	Data Analysis	10
14	Assay Validation	12
15	Limitations of the Method	12
16	Troubleshooting	12
17	Kit Performance	14
17.1	Diagnostic Sensitivity and Specificity	14
17.2	Analytical Sensitivity	14
17.3	Analytical Specificity	15
18	Abbreviations and Symbols	16
19	Literature	16

1 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 real time RT-PCR in the capillary system of the LightCycler® 1.5 or 2.0 (Roche Diagnostics).

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

The virellaEntero 2.0 LC real time RT-PCR Kit contains specific primers and dual-labeled probes for the amplification and 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 Enterovirus specific fragments are performed in an onestep RT-PCR. The amplification can be detected when specific probes are hydrolysed by the polymerase. The emitted fluorescence is measured in the 530 nm channel (F1 of LightCycler®).

Furthermore, virellaEntero 2.0 LC real time RT-PCR 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 specimen.

The amplification of the Control RNA is measured in the 705 nm channel (F3 of LightCycler® 1.5, F6 of LightCycler® 2.0).

4 Package Contents

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
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. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument LightCycler® 1.5 or 2.0
- LightCycler® Capillaries
- Optional: Liquid handling system for automation
- Optional: VLP-RNA (Virus-Like Particles, please look at chapter 'Control RNA' for details)

6 Transport, Storage and Stability

The virellaEntero 2.0 LC real time RT-PCR Kit 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

- 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.

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.
- Regularly decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine virellaEntero 2.0 LC real time RT-PCR Kit components of different lot numbers.

9 Sample Material

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

10 Sample Preparation

The virellaEntero 2.0 LC real time RT-PCR Kit 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:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

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 RNA extraction kit's 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) are not supplied.

Control RNA or VLP-RNA used as Extraction Control:

virellaEnter0 2.0 LC real time RT-PCR Kit 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.

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 PCR set up.
- In every PCR run at least 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 **1:100** in PCR grade water (e.g. 1 μl Control RNA + 99 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 Control RNA* (diluted 1:100)	0.2 μl x (N+1)*
0.2 μl Enzyme	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

- Place the number of capillaries needed into the respective tray of the real time PCR instrument.
- Pipet **16 μl** of the Master Mix into each capillary.
- 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 capillary (Table 4).
- Close the capillaries 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

Description	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	48°C	1
Initial Denaturation	2 min	95°C	1
Amplification			
Denaturation	5 sec	95°C	45
Annealing	20 sec	55°C	
Extension	10 sec	72°C	
		Acquisition mode SINGLE	
Cooling	5 sec	40°C	1

Ramping time for all steps 20°C/sec.

Table 6: Overview of the instrument settings required for the virellaEntero 2.0 LC real time RT-PCR Kit.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler® 1.5	Enterovirus	F1 (530 nm)	Color Compensation Kit needed, e.g. LightCycler® Color Compensation Set
	Control RNA	F3 (705 nm)	
LightCycler® 2.0	Enterovirus	F1 (530 nm)	Color Compensation Kit needed, e.g. LightCycler® Color Compensation Set
	Control RNA	F6 (705 nm)	

13 Data Analysis

The virus specific amplification is measured in the 530 nm channel (F1 of LightCycler® 1.5 and 2.0). The amplification of the Control RNA is measured in the 705 nm channel (channel F3 of LightCycler® 1.5; channel F6 of LightCycler® 2.0).

Following results can occur:

- **A signal in F1 is detected:**
The result is positive, the sample contains Enterovirus RNA.
In this case, detection of a signal of the Control RNA in F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the Control RNA.
- **No signal in F1, but a signal in F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) is detected:**
The result is negative, the sample does not contain Enterovirus RNA.
The signal in channel F3 (LC 1.5) or F6 (LC 2.0) 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 occurred, which can lead to negative results in weak positive samples (see chapter ‚Troubleshooting‘).
- **Neither in F1 nor in F3 (LightCycler® 1.5) or F6 (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 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.

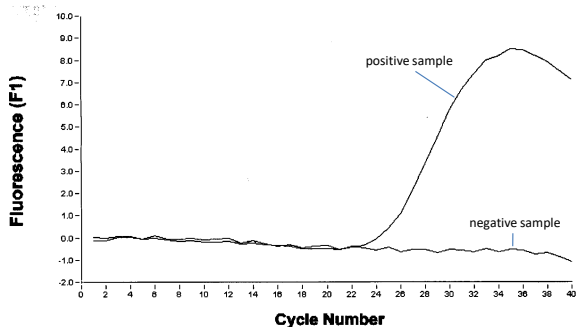


Figure 1: The positive sample shows virus-specific amplification in the F1 channel, whereas no fluorescence signal is detected in the negative sample.

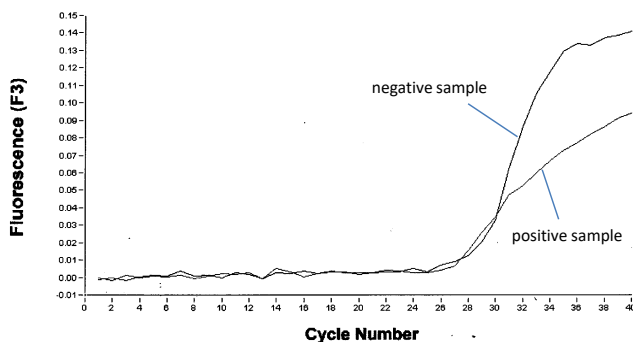


Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA-specific channel (F3 or F6). The amplification signal of the Control RNA in the negative sample shows that the missing signal in the virus-specific channel (F1) is not due to real time RT-PCR inhibition or failure 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. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the F1 channel of the Positive Control

The selected channel for analysis does not comply with the protocol

Select channel F1 for analysis of the virus specific amplification and channel F3 (LightCycler® 1.5) or F6 (LightCycler® 2.0) 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 7.

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (Table 5, page 9).

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 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 chapter '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 F1 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.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the virellaEntero 2.0 LC 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
Enterovirus positive	65	0
Enterovirus negative	0	30
Sensitivity	100%	
Specificity	100%	

17.2 Analytical Sensitivity

The limit of detection (LoD) of virellaEntero 2.0 LC was determined using serial dilutions of Enterovirus cell culture supernatants in culture medium. Total nucleic acids were extracted using NukEx 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 virellaEntero 2.0 LC for *Enteroviruses* is ≤ 0.2 TCID 50 per reaction each.

Table 8: Strains tested for the validation of the sensitivity of virellaEntero 2.0 LC.

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

17.3 Analytical Specificity

The specificity of virellaEntero 2.0 LC was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:





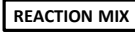










The virellaEntero 2.0 LC real time RT-PCR showed a positive result for the samples containing *Enteroviruses*, whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 8.

Furthermore QCMD and INSTAND ring trials from 2010 – 2015 has been passed successfully with a score of 100% correct results each.

Table 8: Bacterial and viral pathogens tested for the determination of the analytical specificity of virellaEntero 2.0 LC.

Pathogen	virellaEntero 2.0 LC	Pathogen	virellaEntero 2.0 LC
<i>Coxsackievirus A7</i>	positive	<i>Poliovirus 2</i>	positive
<i>Coxsackievirus A9</i>	positive	<i>Poliovirus 3</i>	positive
<i>Coxsackievirus A16</i>	positive	<i>Influenza A Virus</i>	negative
<i>Coxsackievirus A24</i>	positive	<i>Parvovirus 3</i>	negative
<i>Coxsackievirus B3</i>	positive	<i>Norovirus</i>	negative
<i>Coxsackievirus B4</i>	positive	<i>Rotavirus</i>	negative
<i>Coxsackievirus B5</i>	positive	<i>Adenovirus</i>	negative
<i>Coxsackievirus B6</i>	positive	<i>Salmonella thyphimurium</i>	negative
<i>Echovirus 9</i>	positive	<i>Citrobacter freundii</i>	negative
<i>Echovirus 11</i>	positive	<i>Yersinia enterocolitica</i>	negative
<i>Echovirus 20</i>	positive	<i>Listeria monocytogenes</i>	negative
<i>Echovirus 30</i>	positive	<i>Shigella boydii</i>	negative
<i>Enterovirus 71</i>	positive	<i>Shigella sonnei</i>	negative
<i>Enterovirus 68</i>	positive	<i>Shigella flexneri</i>	negative
<i>Poliovirus 1</i>	positive	<i>E. coli</i>	negative

18 Abbreviations and Symbols

RNA	Ribonucleic Acid		Catalog number
PCR	Polymerase Chain Reaction		Contains sufficient for <n> test
RT	Reverse Transcription		Upper limit of temperature
cDNA	Complementary Deoxyribonucleic Acid		Manufacturer
	Reaction Mix		Use by YYYY-MM
	Enzyme		Batch code
	Positive Control		Content
	Negative Control		Consult instructions for use
	Control RNA		<i>In vitro</i> diagnostic medical device
			European Conformity

19 Literature

- [1] Hurst, NP, et al.; (1983) "Coxsackie B infections and arthritis" British Medical Journal, Vol 286, Feb 19, 1983.
- [2] Palmer, AL, et al; (1997) "Adverse Effects of Maternal Enteroviral infection on the Fetus and Placenta" JID 1997;176 (December.)
- [3] For more information: Scott, LA, Stone, MS; (2003), "Viral exanthems," Dermatology Online Journal 9 (3): 4
- [4] Kim K, Hofling K, Carson, SD, Chapman NM, Tracy S; (2003) "The Primary Viruses of Myocarditis," Myocarditis from Bench to Bedside, LT Cooper, ed. (Humana Press) pp23-54.