Manual

MutaPLEX® Enterovirus real time RT-PCR kit

Test for the in vitro detection of enterovirus RNA enterovirus, coxsackievirus, echovirus, poliovirus in clinical and environmental samples

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REF **KG190232**

REF **KG190296**

Σ 32

Σ 96

-20 °C

IVD

CE

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Table of Contents

1 INTENDED USE 2

2 PATHOGEN INFORMATION 2

3 PRINCIPLE OF THE TEST 2

4 PACKAGE CONTENTS 3

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER 3

6 TRANSPORT, STORAGE AND STABILITY 3

7 IMPORTANT NOTES 4

8 GENERAL PRECAUTIONS 4

9 SAMPLE MATERIAL 4

10 SAMPLE PREPARATION 4

11 CONTROL RNA 5

12 REAL TIME RT-PCR 5

12.1 Important points before starting 5
12.2 Procedure 6
12.3 Instrument settings 7

13 DATA ANALYSIS 8

14 ASSAY VALIDATION 10

15 LIMITATIONS OF THE METHOD 10

16 TROUBLESHOOTING 10

17 KIT PERFORMANCE 12

17.1 Diagnostic sensitivity and specificity 12
17.2 Analytical sensitivity 12
17.3 Analytical specificity 13

18 ABBREVIATIONS AND SYMBOLS 14

19 LITERATURE 15

1 INTENDED USE

The MutaPLEX® Enterovirus real time RT-PCR 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 open real time PCR systems.

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 MutaPLEX® Enterovirus real time RT-PCR kit contains specific primers and dual-labelled probes for the amplification and detection of enterovirus RNA (enterovirus, coxsackievirus A and B, echovirus, poliovirus type 1–3) 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 one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the Polymerase. The emitted fluorescence is measured in the FAM channel.

Furthermore, MutaPLEX® Enterovirus real time RT-PCR kit contains a control RNA, which is detected in a second amplification system. Added during RNA extraction, the Control RNA 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 fluorescence of the Control RNA is measured in the VIC®/HEX/JOE™/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 32 (KG190232) or 96 (KG190296) reactions, respectively.

Table 1: Components of the MutaPLEX® Enterovirus 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. MutaCLEAN® Universal RNA/DNA, KG1038, or MutaCLEAN® Mag RNA/DNA, KG1023)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortex mixer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: VLP-RNA (virus-like particles, please see chapter 11 for details).

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® Enterovirus real time RT-PCR-Kit is shipped on dry ice or cool packs. All components must be stored at maximum -20 °C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Opened reagents can be stored at 2–8 °C for up to 6 months.

Up to 20 freeze and thaw cycles are possible. Protect kit components from direct sunlight during the complete test run.

7 IMPORTANT NOTES

- The MutaPLEX® Enterovirus real time RT-PCR 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 instructions 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 MutaPLEX® Enterovirus real time RT-PCR-Kit components of different lot numbers.

9 SAMPLE MATERIAL

Starting material for the MutaPLEX® Enterovirus real time RT-PCR is viral RNA isolated from clinical specimens or environmental samples.

10 SAMPLE PREPARATION

The MutaPLEX® Enterovirus real time RT-PCR is suitable for the detection of enterovirus RNA isolated from clinical specimens with appropriate isolation methods.

Commercial kits for RNA isolation such as MutaCLEAN® Universal RNA/DNA (KG1038) or the magnetic particle-based MutaCLEAN® Mag RNA/DNA (KG1023) are recommended.

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 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.

a) Control RNA or VLP-RNA used as extraction control

MutaPLEX® Enterovirus 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

- 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 – except the enzyme – should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the reaction mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

- We recommend to keep reagents and samples at 2–8 °C (e. g. on ice or a cooling block) at all times.

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. The sensitivity of the detection system is not impaired.

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

Description	Time	Temperature	No of cycles
Reverse Transcription	20 min	45 °C	1
Initial Denaturation	2 min	95 °C	1
Amplification			45
Denaturation	5 s	95 °C	
Annealing	20 s	55 °C	
	Aquisition at the end of this step		
Extension	10 s	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 MutaPLEX® Enterovirus real time RT-PCR.

Real time RT-PCR Instrument	Parameter	Detection Channel	Notes	
LightCycler® 480 I	Enteroviruses Control RNA	483-533 523-568	pre-installed universal CC FAM (510) - VIC (580)	
LightCycler® 480 II	Enteroviruses Control RNA	465-510 533-580		
Stratagene Mx3000P Mx3005P	Enteroviruses Control RNA	FAM HEX	Gain 8 Gain 1	Reference Dye: None
ABI 7500	Enteroviruses Control RNA	FAM JOE	Option Reference Dye ROX: No	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Enteroviruses Control RNA	Green Yellow	Gain 5 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.

The 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/JOETM/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/JOETM/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 chapter „Troubleshooting“).

- **Neither in the FAM nor in the VIC®/HEX/JOETM/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.

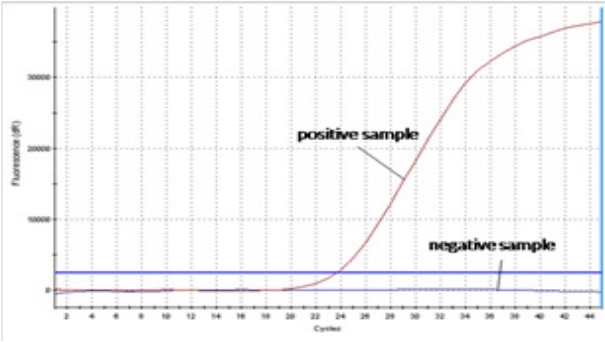

Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

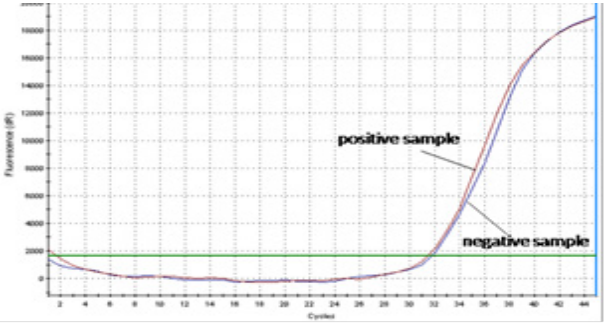

Figure 2: The positive sample as well as the negative sample show a signal in the control RNA-specific VIC®/HEX/JOE™/TET channel. The amplification signal of the control RNA in the negative sample shows that the missing signal in the virus-specific FAM channel is not due to RT-PCR inhibition or failure 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. If you have further questions, please do not hesitate to contact our scientists on info@immundiagnostik.com.

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 channel for the amplification of the control RNA.

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 (table 5).

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 chapter "Transport, Storage and Stability".

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 (chapter 12).

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 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 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 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 MutaPLEX® Enterovirus real time RT-PCR kit, 65 positive and 30 negative samples previously characterized by virus isolation in cell cultures, were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 % (table 7).

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
MutaPLEX® Enterovirus positive	65	0
MutaPLEX® Enterovirus negative	0	30
Sensitivity	100 %	
Specificity	100 %	

17.2 Analytical sensitivity

The limit of detection (LoD) of the MutaPLEX® Enterovirus real time RT-PCR kit was determined using serial dilutions of enterovirus cell culture supernatant in culture medium. Total nucleic acids were extracted using MutaCLEAN Universal 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 the MutaPLEX® Enterovirus real time RT-PCR kit for enterovirus is <0.2 TCID 50 per reaction each.

Table 8: Strains tested for the validation of the sensitivity of the MutaPLEX® Enterovirus real time RT-PCR kit.

Strain	TCID50/ml	Dilution/LoD	corresponding TCID 50
Coxsackievirus A9	1.25×10^{7}	1×10^{-6}	0.2

Strain	TCID50/ml	Dilution/LoD	corresponding TCID 50
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 MutaPLEX® Enterovirus real time RT-PCR was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The MutaPLEX® Enterovirus 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 MutaPLEX® Enterovirus real time RT-PCR.

Strain	Expected Result	Result
Coxsackievirus A7	positive	positive
Coxsackievirus A9	positive	positive
Coxsackievirus A16	positive	positive
Coxsackievirus A24	positive	positive
Coxsackievirus B3	positive	positive
Coxsackievirus B4	positive	positive
Coxsackievirus B5	positive	positive
Coxsackievirus B6	positive	positive
Echovirus 9	positive	positive
Echovirus 11	positive	positive
Echovirus 20	positive	positive
Echovirus 30	positive	positive
Enterovirus 71	positive	positive
Enterovirus 68	positive	positive

Strain	Expected Result	Result
Poliovirus 1	positive	positive
Poliovirus 2	positive	positive
Poliovirus 3	positive	positive
Influenzavirus A	negative	negative
Parechovirus 3	negative	negative
Norovirus	negative	negative
Rotavirus	negative	negative
Adenovirus	negative	negative
Salmonella thyphimurium	negative	negative
Citrobacter freundii	negative	negative
Yersinia enterocolitica	negative	negative
Listeria monocytogenes	negative	negative
Shigella boydii	negative	negative
Shigella sonnei	negative	negative
Shigella flexneri	negative	negative
Escherichia coli	negative	negative

18 ABBREVIATIONS AND SYMBOLS

cDNA	Complementary deoxyribonucleic acid		Catalog number
C_T	Cycle Threshold		To be used with
PCR	Polymerase chain reaction		Contains sufficient for <n> test
RNA	Ribonucleic acid		Upper limit of temperature
RT	Reverse transcription		Manufacturer
VLP	Virus-like particles		Use by

REACTION MIX	Reaction mix	LOT	Lot number
ENZYME	Enzyme	CONT	Content
CONTROL +	Positive control		Consult instructions for use
CONTROL –	Negative control	IVD	*In vitro* diagnostic medical device
CONTROL RNA IC	Control RNA		

19 LITERATURE

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