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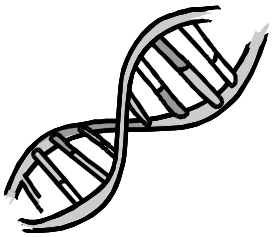
MutaPLEX® Enterovirus

real time RT-PCR Kit



Screening assay for the *real time* detection of human enterovirus RNA (Polio-, Coxsackie A-, Coxsackie B- and Echovirus) in clinical samples using open (e.g. microtiterplate) *real time* PCR systems (e. g. Applied Biosystems ABI, Corbett Research: RotorGene, Cepheid: Smart Cycler, Stratagene or Light Cycler 480, Roche) and parallel control of RNA extraction efficiency.

REF IMM-KG190232 Σ 32

REF IMM-KG190296 Σ 96

-20°C

For in vitro diagnostic use only

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1. INTENDED USE

The **MutaPLEX® Enterovirus** *real time* RT-PCR kit is a qualitative assay for the specific detection of enterovirus (Polio-, Coxsackie A-, Coxsackie B- and Echoviruses) RNA in stool or other clinical sample matrices (whole blood, plasma, respiratory samples, CSF [cerebrospinal fluid] etc.) using open real time RT-PCR systems (e.g. from ABI, Stratagene, Corbett Research: RotorGene, Cepheid: Smart Cycler, or Light Cycler 480 (Roche). In parallel, the RNA extraction efficiency from stool samples is controlled by a separate internal control.

2. INTRODUCTION

Human enteroviruses are ubiquitous high-infectious pathogens belonging to the family of Picornavirida. They have a high incidence worldwide (about 500 million infections/ year). The non-enveloped viruses (with ss-RNA genome of 6-7 kb) are high resistant even against pH values from 3-9. Thus far, there is no effective antiviral chemotherapeutics available. Until now about 70 serotypes are known. Route of transmission in human is fecal-oral by contact and smear infection. Contaminated food and water (also knifes and forkes) are an important source of infection for this highly contagious viruses which can be secreted with stool from infected patients for several weeks.
Enteroviruses may trigger many (even life-threatening) infections, especially among children. In summer period very often contaminated waters (swimming pools, lakes) change for the worse. Enteroviruses cause several diseases, e.g. infection of upper respiratory tract, summer flue, herpangina, hand-foot-mouth-disease, juvenile diabetes mellitus, infection of gastrointestinal tract, eye-infections or epidemic myalgie. But also myocarditis, paralysis, multiple organ failure, meningitis and encephalitis may be associated with enterovirus infections.

3. PRINCIPLE OF THE TEST

MutaPLEX® Enterovirus *real time* RT-PCR kit contains specific primers, fluorescence-marked probes and additional material for the detection of enterovirus RNA in clinical samples. Human stool or other clinical samples, such as whole blood, plasma, respiratory samples, CSF (cerebrospinal fluid) etc. can be used as starting material for RNA-extraction and following pathogen analysis with **MutaLPLEX® Enterovirus.**

Reverse transcription (RT) of potentially contained viral RNA to cDNA and subsequent amplification of enterovirus-specifc fragments by means of PCR is done in only **one step**. Target sequence for the detection is within the 5`-untranslated region (5`-UTR) of the genome.
Detection of enterovirus amplificates is achieved in *real time* by hybridization and subsequent hydrolysis of enterovirus-specific fluorescence probes. Their emitted signal is measured (during PCR process) by the optical unit of the *real time* PCR system in use (ABI: PRISM SDS-, Stratagene: MxPRO-, Corbett Research: RotorGene 3000/ 6000-, Cepheid: SmartCycler-Software, Roche: LC480- software). **Enterovirus-specific amplification** is measured in **FAM**-channel (**470**/ **510** nm).

Furthermore, **MutaPLEX® Enterovirus** *real time* RT-PCR - kit contains also a separate RNA extraction control. This is added initially prior to the nucleic acid extraction procedure and detected in the follow-up by an independent amplification step. This internal control (IC) allows on the one hand the exposure of errors during RNA extraction procedure and identifies on the other hand a potential inhibition of reverse transcription or PCR. Doing this, the risk for false-negative results is minimized extensively. **RNA extraction control** (= internal control for PCR) is measured in **VIC**- (respectively = **HEX/ JOE/ TET/ Cy3)** channel (**530**/ **555** nm).

4. KIT CONTENT

Each kit contains enough reagents to perform **32** or **96** tests and also a package insert.

	Reagent	Confection 32	Confection 96	Colour
A1	Enzyme Mix	1 x 30 µl	3 x 30 µl	**blue**
A2	Primer-/ Probe Mix	1 x 500 µl	3 x 500 µl	**yellow**
A3	Positive Control	1 x 30 µl	2 x 30 µl	**red**
A4	Negative Control	1 x 200 µl	1 x 200 µl	**green**
A5	Extraction Control (IC)	1 x 200 µl	3 x 200 µl	transparent

5. TEST PERFORMANCE

Required materials - provided:

- Reagents for *real time* RT-PCR
- Package insert

Required materials - not provided:

- *Real time* PCR instrument (e.g. from ABI, Stratagene, Corbett Research: Rotor Gene, Cepheid: Smart Cycler or Roche: Light Cycler 480)
- PCR reaction tubes (or optical microtiter plates with transparent optical covers)
- Table centrifuge (13000 rpm) and Cryo container for PCR tubes
- RNA extraction kit for stool samples (e.g. **MutaCLEAN® Stool (Viral RNA),** KG1035, **Immundiagnostik**)
- Pipets (0.5 µl – 1000 µl) with sterile filter tips
- sterile reaction tubes (1,5 ml and 2,0 ml)

6. STORAGE AND HANDLING

- All reagents (A1 to A5) should be stored **until immediate use** at **<-20°C**.
- **Avoid several freeze/ thawing cycles** for reagents A2, A3 and A5 (in case of sporadic use prepare **suited aliquots**).
- **Cool** all reagents during the working steps.
- Store Primer-/ Probe-Mix (A2) in the dark to **protect** the fluorescence probes **from light**.
- All reagents can be used until the expiration date printed on the labels.

7. WARNINGS AND PRECAUTIONS

- For in vitro diagnostic use only.
- This assay needs to be carried out by in Molecular Biology methods skilled personnel.
- Clinical samples should be regarded as potentially infectious materials.
- This assay needs to be run according to GLP (Good Laboratory Practice).
- Do not use the kit after its expiration date.

AMPLIFICATION: The PCR technology is utmost sensitive. Thus, amplification of a single molecule generates millions of identical copies. These copies may evade through aerosols and sit on surfaces. In order to avoid contamination of samples with RNA which previously was amplified, it is important to physically strictly divide sample and reagent preparation units from sample amplification units. Set up three separate working areas:

1) Area for sample preparation.
2) Area for preparation of Master Mix.
3) Area for pipetting Master Mix and samples to the PCR tubes.

Pipets, vials and other working materials should not circulate among working units!

- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Avoid aerosols.
- Routinely decontaminate your pipettes and laboratory benches with decontaminant (do not use ethanol solutions).

8. PROCEDURE

The complete procedure is separated in three steps:

A) RNA extraction from stool samples (e.g. with **MutaCLEAN® Stool (Viral RNA)**, KG1035 from **Immundiagnostik**) or other clinical samples using a commercial extraction kit.

B) Reverse transcription (RT) of RNA and subsequent amplification/ combined detection of generated cDNA templates using hybridisation probes.

C) Interpretation of the results with the software of the real time PCR instrument in use.

A) SAMPLE PREPARATION

1) **RNA Extraction** (by use of a commercial available RNA isolation kit): Extract viral RNA from stool (e. g. with **MutaCLEAN® Stool (Viral RNA)**, KG1035 from **Immundiagnostik**) or from other clinical sample matrices by use of a commercial RNA isolation kit (suited for whole blood, plasma, respiratory samples, CSF [cerebrospinal fluid] etc.) according to the manufacturer`s instructions. For doing this:

a) **Stool Samples**: resuspend a “pea-sized” stool sample in 1.5 ml **sterile water** (do **not** use buffers!) by intensive vortexing and let sediment solid particles (if necessary support by centrifugation for 2 min at 5000 rpm in table centrifuge). Use the supernatant for RNA extraction without any further dilution. Very liquid stool samples should be used directly (without further resuspension) for the RNA extraction, but also here vortex intensively and proceed as before described.

b) **Liquor**: Liquor samples can be used directly (“native”) for extraction procedure.

c) **Swab**: soak the pad in 500 µl **sterile water** (do **not** use buffers!) and resuspend thoroughly by vortexing. Remove pad-device and use remaining solution with eluted RNA for the extraction procedure.

2) **Important**: independent from the used sample matrix also a water control should be extracted additionally: this is for identification of possible occurrence of inhibiting matrix effects. Treat this water control analogous to the samples.

3) The extraction control A5 from **MutaREX® Enterovirus** is added to each sample BEFORE begin of the extraction procedure: Prepare a “working solution“ with first buffer of the used RNA isolation kit (eventually plus supplements as „Poly A“) and the extraction control (A5). Multiply the buffer volume necessary for one extraction (e.g. 200 µl for

MutaCLEAN® Stool (Viral RNA) from **Immundiagnostik**) with number (N) of extractions performed in total (do not forget the water control and add also one additional sample volume for reasons of inaccurate pipetting) and add for each extraction 5 µl of A5:
= (extraction buffer volume + 5 µl A5) x (N+1). Perform subsequent extraction procedure according to the manufacturer`s manual afterwards.

OPTIONAL: If no control of extraction efficiency is needed, but still an internal control (IC) of PCR, reagent A5 must be added (during preparation of PCR) directly to the PCR Master Mix. In that case use only **0.2 µl** from a **1:10 dilution of A5** per reaction!

4) If *real time* RT-PCR is not performed immediately, store the extracted RNA at **< -20°C**.

B) *Real time* Enterovirus (MutaPLEX®) RT-PCR-PROTOCOL

Please **read** carefully the complete protocol **before** starting procedure! Each assay should include a negative and positive control. Use filter tips for all pipetting steps. The necessary total Master Mix volume is calculated as follows:

1) Multiply Enzyme Mix volume per reaction with the number (n) of necessary reactions (samples and controls) + add one additional sample volume for reasons of inaccurate pipetting; proceed in the same manner with all additional reagents!
Cool all reagents during the working steps!

2) For **PCR Master Mix** preparation combine the following reagents in a sterile tube and mix gently by pipetting (about **15 - 20x !** - **do NOT vortex!**):

Reaction Volume	Master Mix Volume
0.8 µl Enzyme Mix (A1)	**0.8 µl** x (n+1)
14.2 µl Primer-/ Probe Mix (A2)	**14.2 µl** x (n+1)

3) Pipet **15 µl** of this Master Mix using micropipets with sterile filter tips in each of necessary PCR reaction tubes (or wells of an optical 96 well microtiter plate); do not forget the controls.

Add **5 µl** of the RNA sample, the positive (A3), the negative (A4) and the water control to each of the corresponding PCR reaction tube. Mix by pipetting several times up and down. It is recommended to close the tubes immediately after filling and to prepare the negative control indeed first but to close its tube last (as contamination control). In case optical microtiter plates are used, cover the used wells with an optical adhesive foil.

4) Transfer the reaction tubes into the *real time* PCR system and run the RT-PCR using following temperature protocol:

50°C for **10 min**		*reverse transcription*
95°C for **10 min**		*initial denaturation*
95°C for **15 sec**		**45 cycles**
53°C for **30 sec**		ramping time: 20°C/sec – aqu. mode here: SINGLE
72°C for **15 sec**		
4°C for **30 sec**		*cooling*

C) RT-PCR ANALYSIS AND INTERPRETATION OF RESULTS

1) Performance of *real time* RT-PCR with **MutaPLEX® Enterovirus**.

2) The result of enterovirus specific amplification is shown in the **FAM**-channel (source 470 nm/ detection **510 nm**) and the result of the extraction efficiency control (= internal control **IC**) is shown in **VIC**- (= HEX/ JOE/ TET/ Cy3) channel (source 530 nm/ detection **555 nm**).

3) Use following settings to define a reporter + quencher with the ABI PRISM SDS software:

Detection	Reporter	Quencher
Enterovirus RNA	**FAM**	none
Internal Control	**VIC** / HEX / JOE	none

Following results can occure:

1) FAM fluorescence is detected.
The result is positive**: The sample contains enterovirus RNA.**
The occurence of VIC/ HEX/ JOE/ TET/ Cy3 fluorescence is inessential as high concentrations of *Enterovirus* RNA may reduce or even inhibit the amplification of the internal control.

2) No FAM, but VIC / HEX / JOE fluorescence is detected.
The result is negative: **The sample does not contain any enterovirus RNA.**
The detected signal of the internal control excludes the possibility of an inhibition of the RT-PCR.

3) Neither FAM, nor VIC / HEX / JOE fluorescence is detected.
A diagnostic statement can not be made.
An **inhibition** of the RT-PCR reaction occurred.