Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Enterovirus Real-TM

HANDBOOK

Real Time PCR Kit for the qualitative detection of Enterovirus RNA

REF V16-50FRT

REF TV16-50FRT

Σ 50

NAME

Enterovirus Real-TM

INTRODUCTION

Enteroviruses are a genus of (+)ssRNA viruses associated with several human and mammalian diseases. Serologic studies have distinguished 66 human enterovirus serotypes on the basis of antibody neutralization tests. Additional antigenic variants have been defined within several of the serotypes on the basis of reduced or nonreciprocal cross-neutralization between variant strains. On the basis of their pathogenesis in humans and animals, the enteroviruses were originally classified into four groups, polioviruses, Coxsackie A viruses (CA), Coxsackie B viruses (CB), and echoviruses, but it was quickly realized that there were significant overlaps in the biological properties of viruses in the different groups. Enteroviruses affect millions of people worldwide each year, and are often found in the respiratory secretions (e.g., saliva, sputum, or nasal mucus) and stool of an infected person. Historically, poliomyelitis was the most significant disease caused by an enterovirus, Poliovirus. There are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses. Poliovirus, as well as coxsackie and echovirus are spread through the fecal-oral route. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, severe neonatal sepsis-like disease, and acute flaccid paralysis.

INTENDED USE

Kit **Enterovirus Real-TM** is a Real-Time test for the qualitative detection of *Enterovirus RNA* in the biological materials and in the environment. RNA is extracted from specimens, amplified using one step RT-amplification and detected using fluorescent reporter dye probes specific for Enterovirus RNA and IC (Internal Control).

PRINCIPLE OF ASSAY

Kit **Enterovirus Real-TM** is based on three major processes: isolation of RNA from specimens, reverse transcription of the RNA and Real Time amplification

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V16-50FRT)

Part N° 2 – **"Enterovirus Real-TM":** Real Time amplification kit

- **PCR-mix-1 *Enterovirus***, 0.6 ml;
- **PCR-mix-2-FRT,** 0.3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Reverse transcriptase (M-MLV)**, 0,015 ml ;
- **RT-G-mix-2,** 0,015 ml;
- **Positive Control *Enterovirus cDNA* C+,** 0,2 ml;
- **DNA-buffer**, 0,2 ml;
- **Negative Control*** 1,2 ml;
- **Internal Control RNA (IC RNA)****, 0.5 ml.

Contains reagents for 55 reactions

Module No.2: Complete Real Time PCR test with RNA purification kit (TV16-50FRT)

Part N° 1 – **"Ribo-Sorb":** Sample preparation

- **Lysis Solution**, 22,5 ml;
- **Washing Solution**, 20 ml;
- **Sorbent**, 1,25 ml.
- **RNA-eluent**, 5 x 0,5 ml;

Contains reagents for 50 tests.

Part N° 2 – **"Enterovirus Real-TM":** Real Time amplification kit

- **PCR-mix-1 *Enterovirus***, 0.6 ml;
- **PCR-mix-2-FRT,** 0.3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Reverse transcriptase (M-MLV)**, 0,015 ml ;
- **RT-G-mix-2,** 0,015 ml;
- **Positive Control *Enterovirus cDNA* C+,** 0,2 ml;
- **DNA-buffer**, 0,2 ml;
- **Negative Control*** 1,2 ml;
- **Internal Control RNA (IC RNA)****, 0.5 ml.

Contains reagents for 55 reactions

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- RNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 5°C dry heat block
- Vortex mixer
- Pipettors with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone
- Refrigerator, Freezer

Zone 2: RT and amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Refrigerator, Freezer

STORAGE INSTRUCTIONS

The whole **“Enterovirus Real-TM”** kit must be stored at **-20°C**. The whole “**Ribo-Sorb”** kit must be stored at **2-8°C**. Kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Enterovirus Real-TM kit is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Enterovirus Real-TM can analyze RNA extracted from:

- *Cerebrospinal fluid* (ready for extraction) - 0,1 ml;
- *water:* centrifuge 10-20 ml for 10 min at maximum speed. Discard the supernatant and leave about 100 µl of solution for RNA extraction;
- *whole blood* collected in EDTA tubes*;*
- *feces:*
    - Prepare 10-20% feces suspension, for instance adding 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces in 5 ml tube (the same can be done in 2,0 ml tube). The DNA/RNA purification must be done immediately, if it is not possible add 20% Glycerol sterile solution (cryoprotective agent that provides intracellular and extracellular protection against freezing) and store at -20°C.
    - Vortex to get an homogeneous suspension and centrifuge for 5 min to 7000-12000g. Use the supernatant for the extraction of the viral DNA/RNA.

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.
Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.
Sacace Biotechnologies recommends to use the following kits:

⇒ **Ribo-Sorb-** (Sacace, REF K-2-1)

⇒ **Ribo Virus** spin column extraction kit (Sacace, REF K-2-C)

⇒ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003): for CFS and cell free body fluids.

Please carry out the RNA extraction according to the manufacturer’s instructions. Add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **450 µl Lysis Solution** and **10 µl** of **IC RNA**.
3. Add **100 µl** of samples to the appropriate tube containing Lysis Solution and IC. Mix by pipetting and incubate 5 min at room temperature.
4. Prepare Controls as follows:
   - add **100 µl** of **Negative Control** to the tube labeled *Cneg*.
5. Vortex the tubes and centrifuge for 5 sec at 5000g. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 1 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for RNA/DNA extraction
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
8. Centrifuge all tubes for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
9. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **500 µl** of **Ethanol 70%** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Repeat step 10.
12. Add **400 µl** of **Acetone** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
13. Incubate all tubes with open cap for 10 min at 60°C.
14. Resuspend the pellet in **50 µl** of **RNA-eluent.** Incubate for 10 min at 60°C and vortex periodically. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g).
15. The supernatant contains RNA ready for use. The RT-PCR can be performed the same day. If this is not possible, the RNA preparations can be stored at -80°C for up to one month.

REVERSE TRANSCRIPTION AND REAL TIME AMPLIFICATION (25 ul total vol):

1. Prepare the reaction mix just before use. Prepare the reaction mixture for required number of reactions, including clinical and control samples as specified in Table 1.
2. Prepare required quantity of reaction tubes (or PCR plate) for clinical and control samples.
3. To prepare the reaction mix add to a new sterile tube **PCR-mix-1 *Enterovirus,* PCR-mix-2-FRT**, **RT-G-mix-2, TaqF Polymerase** and **Reverse transcriptase (M-MLV)** according to **Table 1**. Vortex the tubes thoroughly.
4. Transfer **15 µl** of reaction mix to each tube.

Table 1. **Preparation of reaction mix**

		Reagent volume for the specified number of reactions, µl				
Reagent volume for 1 reaction (µl)		10.00	5.00	0.25	0.50	0.25
Number of samples	Number of reactions	PCR-mix-1 Enterovirus	PCR-mix-2-FRT	RT-G-mix-2	TaqF Polymerase	Reverse transcriptase (M-MLV)
2	6	60	30	1.5	3.0	1.5
4	8	80	40	2.0	4.0	2.0
6	10	100	50	2.5	5.0	2.5
8	12	120	60	3.0	6.0	3.0
10	14	140	70	3.5	7.0	3.5
12	16	160	80	4.0	8.0	4.0
14	18	180	90	4.5	9.0	4.5
16	20	200	100	5.0	10.0	5.0
18	22	220	110	5.5	11.0	5.5
20	24	240	120	6.0	12.0	6.0
22	26	260	130	6.5	13.0	6.5
24	28	280	140	7.0	14.0	7.0
26	30	300	150	7.5	15.0	7.5
28	32	320	160	8.0	16.0	8.0

5. Add **10 µl** of **RNA** extracted from clinical and control samples to the appropriate tube.
6. Prepare for each panel the following controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Negative Control of Amplification (NCA);
   - add **10 µl** of **Positive Control *Enterovirus cDNA* C+,** to the tube labeled Positive Control of Amplification (C+);
   - add **10 µl** of the extracted **Negative Control of Extraction** to the tube labeled Negative Control of Extraction (NCE).

Set the following parameters of amplification:

Reaction Volume (µl): **25**

RT- Amplification

1. Create a temperature profile on your instrument as follows:

Step	Temp, °C	Time	Fluorescence detection	Cycle repeats
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 s	–	45
	60	20 s	FAM(Green), JOE(Yellow)	

For example: SaCycler-96™ (Sacace), iQ5™ (BioRad); Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen), Mx3005P™ (Agilent)

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Internal Control (IC) is detected on the FAM (Green) channel and *Enterovirus cDNA* is detected on the JOE (Yellow).

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate / Gain Optimization …	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*From 5 to 10 FL*	*0.05*	*10 %*	*On*
JOE/Yellow/HEX	*From 5 to 10 FL*	*0.05*	*10 %*	*On*

Plate- or modular type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are in accordance with the table 2

Table 2: results for controls

Control	Stage for control	Ct channel FAM (Green)	Ct channel JOE (Yellow)/HEX/Cy3	Interpretation
NCE	RNA isolation	Pos (< boundary value)	Neg or > boundary value	Valid result
NCA	RT and Amplification	Neg or > boundary value	Neg or > boundary value	Valid result
C+	RT and Amplification	Pos (< boundary value)	Pos (< boundary value)	Valid result

Ct Boundary values for rotor type instruments (RotorGene 3000/6000/Q):

Control Sample	Channel	Boundary Ct value
C+	FAM	38
	JOE/HEX	33
NCE	FAM	38
	JOE/HEX	>38 or absent
NCA	FAM	>38 or absent
	JOE/HEX	>38 or absent
Test samples	FAM	38
	JOE/HEX	38

Ct Boundary values for plate type instruments (SaCycler-96, iQ5, MX3005):

Control Sample	Channel	Boundary Ct value
C+	FAM	40
	JOE/HEX	40
NCE	FAM	40
	JOE/HEX	>40 or absent
NCA	FAM	>40 or absent
	JOE/HEX	>40 or absent
Test samples	FAM	40
	JOE/HEX	40

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Enterovirus* primers and probes. The specificity of the kit **Enterovirus Real-TM** was 100%. The potential cross-reactivity of the kit **Enterovirus Real-TM** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Enterovirus Real-TM** allows to detect *Enterovirus* RNA in 100% of the tests with a sensitivity of not less than 1000 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: 5'UTR

TROUBLESHOOTING

1. Weak (Ct > 38/40) signal of the IC (Fam(Green) channel): retesting of the sample is required.
    - The PCR was inhibited.
        ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
    - The reagents storage conditions didn't comply with the instructions.
        ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 40) signal on the JOE(Yellow)/HEX/Cy3 channel: retesting of the sample is required.
3. Any signal on the Joe/HEX/Cy3 channel with Negative Control of extraction.
    - Contamination during RNA extraction procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        ⇒ Pipette the Positive controls at the end.
        ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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