IVD For *in Vitro* Diagnostic Use

CE

Enterovirus 71-Type Real-TM

Handbook

Real-Time PCR test for qualitative detection of Enterovirus-71 Virus (EV1)

REF V64-50FRT

Σ 50

NAME

ENTEROVIRUS-71 Real-TM

INTRODUCTON

Enterovirus 71 (EV71) is a virus of the genus Enterovirus in the Picornaviridae family notable for its etiological role in epidemics of severe neurological diseases in children. This virus is a member of the enterovirus species A and appears to have evolved only recently with the first known strain isolated in 1965. It has since spread to various countries in Europa (Bulgaria, Hungary) and Asia (Malaysia, Korea, China, Taiwan, Cambodia) where it has been responsible for several outbreaks.

To make a definite diagnosis of EV71 infection, laboratory testing such as PCR or culture for the virus from samples of throat secretions, faeces or CSF (cerebrospinal fluid) is needed.

INTENDED USE

ENTEROVIRUS-71 Real-TM is a Real-Time test for the qualitative detection of Enterovirus-71 Virus RNA in the biological materials and in the environment.. ENTEROVIRUS-71 RNA is extracted from specimens, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for Enterovirus-71 or Internal Control (IC).

PRINCIPLE OF ASSAY

Enterovirus-71 virus detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region by using specific primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run. **ENTEROVIRUS-71 Real-TM Qual** PCR kit is a qualitative test that contains the Internal Control (IC), which must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. **ENTEROVIRUS-71 Real-TM Qual** PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF), which is activated by heating at 95 °C for 15 min.

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FRT *EV71*	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FRT	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
Pos Control cDNA *EV71* / IC (C+)	colorless clear liquid	0.2	1 tube
RNA-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C–)*	straw-colored clear liquid	1.2	1 tube
Internal Control STI-87-rec (IC)**	colorless clear liquid	0.12	5 tubes

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

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Personal Real Time PCR thermocycler.
- Disposable polypropylene microtubes for PCR or PCR-plate**.**
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

STORAGE INSTRUCTIONS

All components of the **ENTEROVIRUS-71 Real-TM** PCR kit (except for RT-G-mix-2, RT-PCR-mix-1-FRT *EV71,* RT-PCR-mix-2-FRT, polymerase (TaqF), and TM-Revertase (MMlv)) are to be stored at 2–8 ºC. All components of the **ENTEROVIRUS-71 Real-TM Qual** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

RT-G-mix-2, RT-PCR-mix-1-FRT *EV71,* RT-PCR-mix-2-FRT, polymerase (TaqF), and TM-Revertase (MMlv) are to be stored at ≤ –16 °C

RT-PCR-mix-1-FRT *EV71* is to be kept away from light.

ENTEROVIRUS-71 Real-TM PCR kit should be transported at 2–8 °C for no longer than 5 days but should be stored at 2-8 and -20°C immediately on receipt.

STABILITY

ENTEROVIRUS-71 Real-TM Test 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. The shelf life of reagents before and after the first use is the same, unless otherwise stated. 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

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:

- 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.

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. 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-71 Real-TM can analyze RNA extracted from:

- *CSF* (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*;*
- *swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 mL of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.
- *tissue* (≈1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *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)

⇒ **DNA/RNA-Prep** (Sacace, REF K-2-9);

⇒ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003);

Please carry out the RNA extraction according to the manufacturer's instructions.

During extraction, use the following controls:

- Negative Control (C–): *add 100 µl of Negative Control (C–) to labeled C-*
- Internal Control STI-87-rec (IC): *add 10 µl of Internal Control RNA during the RNA isolation procedure directly to the sample/lysis mixture.*

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

1. Prepare required quantity of reaction tubes.
2. Prepare for each sample in the new sterile tube **Reaction Mix**: add **10 µl** of **RT-PCR-mix-1**, **5 µl** of **RT-PCR-mix-2, 0,25 µl** of **RT-G-mix-2**, **0,50 µl** of **TaqF Polymerase** and **0,25 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge for 5 sec.

   This mix must be used immediately. Don't store the prepared mix!

Reagents volume x 1 reaction (µl)		**10,0**	**5,00**	**0,25**	**0,50**	**0,25**
N RNA samples[1]	N reactions[2]	RT-PCR-mix-1	RT-PCR-mix-2	RT-G-mix-2	TaqF Polymerase	M-MLV Revertase
4	6	60	30	1,5	3,0	1,5
6	8	80	40	2,0	4,0	2,0
8	10	100	50	2,5	5,0	2,5
10	12	120	60	3,0	6,0	3,0
12	14	140	70	3,5	7,0	3,5
...58	60	600	300	15,0	30,0	15,0

[1] specimens plus 2 extraction controls (N+2)
[2] specimens plus extraction and amplification controls (N+2+2)

3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted RNA** sample to appropriate tubes with Reaction Mix and mix well by pipetting.
5. Prepare for each panel 2 controls:
   - NCA: add **10 µl** of **RT-eluent** to the tube labeled Negative Control of amplification;
   - C+: add **10 µl** of **Pos Control cDNA *EV71* / IC (C+)** to the tube labeled Positive Control;

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

Enterovirus-71 cDNA is detected on the JOE(Yellow)/HEX/Cy3 channel, IC DNA on the FAM (Green) channel

Amplification

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

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	50 °C	30 min	–	1
2	95 °C	15 min	–	1
3	95 °C	10 s	–	45
	60 °C	20 s	FAM/Green, HEX/Cy3/Joe/Yellow	
	72 °C	10 s		

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen), SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™/Mx3000P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)*

Fluorescence is detected at stage 3 (60 °C) in FAM/Green and HEX/Cy3/Joe/Yellow channels.

INSTRUMENT SETTINGS

Rotor-type instruments

Settings

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct	Dynamic tube
FAM/Green	*from 3 Fl to 8 Fl*	0.05	10 %	On	On
JOE/Yellow	*from 3 Fl to 8 Fl*	0.05	10 %	On	On

Plate-type instruments

Settings

Channel	Threshold
FAM	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.
HEX/Joe/Cy3	

DATA ANALYSIS

Accumulation of ***Enterovirus-71 virus*** **cDNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control amplification product is detected in the **FAM/Green** channel.

The results are interpreted by the software of the PCR instrument used by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

Table: Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM/Green	JOE/Yellow/HEX	
NCE	RNA extraction	≤ 38	Neg	OK
NCA	RT-PCR	Neg	Neg	OK
C+	RT-PCR	≤ 38	≤ 38	OK

1. The sample is considered **positive** if its Ct value detected in the JOE/Yellow/HEX channel does not exceed the boundary Ct value (< 40 for clinical samples) and the Ct value detected in the FAM/Green channel does not exceed the value specified for the Internal Control (Ct < 38). The fluorescence curve should have a typical sigmoid shape and cross the threshold line once in the region of significant fluorescence increase.
2. The sample is considered **negative** if its Ct in the JOE/Yellow/HEX channel is not detected (the fluorescence curve does not cross the threshold line) and the Ct value detected in the FAM/Green channel does not exceed the boundary Ct value specified for the Internal Control (Ct < 38).

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

SPECIFICATIONS

Analytical specificity

The analytical specificity of **ENTEROVIRUS-71 Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The clinical specificity of **ENTEROVIRUS-71 Real-TM** PCR kit was confirmed in laboratory clinical tests. 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-71 Real-TM** allows to detect *ENTEROVIRUS-71* RNA in 100% of the tests with a sensitivity of not less than 10^3 copies/ml.

The claimed analytical features of **ENTEROVIRUS-71 Real-TM Qual** PCR kit are guaranteed only when an additional reagent kit (DNA/RNA-prep or RIBO-sorb) is used.

TROUBLESHOOTING

The results of analysis are not taken into account in the following cases:

1. If the Ct value of a clinical sample detected in the JOE/Yellow/HEX channel exceeds the boundary Ct value (>40), the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive Ct value is detected, the sample is considered to be **positive**.
2. If any Ct value is detected for the Negative Control of Amplification (NCA) in both channels or the Ct value is detected for Negative Control of Extraction (C–) in the JOE/Yellow/HEX channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis of all tests and to take measures to detect and eliminate the source of contamination.
3. If the Ct value is absent for the Positive Control of RT-PCR (C+), this indicates errors in carrying out PCR or an incorrect amplification program. RT-PCR should be repeated for all samples.
4. If the Ct value of a clinical sample is absent or greater than the boundary Ct value (>40) for the JOE/Yellow/HEX channel and the Ct value in the FAM/Green channel is greater than the Ct values specified for the Internal Control (>38), the result is **invalid**. Analysis of such samples should be repeated starting from the RNA extraction stage.

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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