IVD For *in Vitro* Diagnostic Use

CE

Poliovirus Real-TM

Handbook

Real Time PCR test for qualitative detection of Poliovirus/*Enterovirus* group C(*HEV-C*) RNA and differentiation of *Poliovirus* strains *Sabin 1, Sabin 2, Sabin 3*

REF V58-50FRT

REF TV58-50FRT

Σ **50**

NAME

Poliovirus Real-TM

INTRODUCTION

Poliomyelitis is an acute paralytic disease caused by poliovirus, a Human Enterovirus C of the Picornaviridae family which is classified into three distinct and stable serotypes.

Since the 1960s, poliomyelitis has been effectively controlled by the use of inactivated or live attenuated vaccines. The Sabin live oral poliovirus vaccine (OPV) is constituted of attenuated strains of each of the three serotypes which have been selected by numerous passages of wild-type strains in monkey tissues in vivo and in vitro. The OPV strains (Sabin 1, 2 and 3) replicate in the human gut and induce a strong and long-lasting immune response, including a local intestine immunity. However, in rare cases (1case per 0.2 to 2.5million doses), OPV strains can cause vaccine-associated paralytic poliomyelitis (VAPP). Reversion of vaccine strains toward a pathogenic phenotype is probably one of the main causes of VAPP, a disease most frequently associated with type Sabin 3 and type Sabin 2 strains and more rarely with the type Sabin 1 strain.

INTENDED USE

Kit **Poliovirus Real-TM** is a Real-Time test for the qualitative detection of *Poliovirus*/*Enterovirus* group C(*HEV-C*) RNA and differentiation of *Poliovirus* strains *Sabin 1, Sabin 2, Sabin 3* in the biological materials and in the environment.

PRINCIPLE OF ASSAY

Kit **Poliovirus Real-TM** is based on three major processes: isolation of RNA from specimens, reverse transcription of the RNA and Real Time amplification. Test contains the Internal Control (IC) which must be used in the RNA purification procedure and serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

Poliovirus detection by the polymerase chain reaction (PCR) is based on the multiplex amplification of the pathogen genome specific region in two tubes using specific primers:

- **PCR-mix-1 *HEV-C*/IC** tube contains primers directed against *Poliovirus* and *Enterovirus* group C (*HEV-C*) RNA and Internal Control (*HEV-C* cDNA is detected in the FAM/Green fluorescence channel and IC is detected in the JOE/Yellow/HEX/Cy3 channel);
- **PCR-mix-1-FL *Sabin* 1/2/3** tube contains primers directed against *Poliovirus* strains *Sabin 1, Sabin 2, Sabin 3 (Sabin 1* cDNA is detected in the ROX/Orange/TexasRed fluorescence channel, *Sabin 2* cDNA is detected in the FAM/Green channel, *Sabin 3* cDNA is detected in the JOE/Yellow/HEX/Cy3 channel).

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V58-50FRT)

Part N° 2 – **"Reverta-L":** Reverse transcription of the RNA

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 3 – **"Poliovirus Real-TM":** Real Time amplification kit

- **PCR-mix-1 *HEV-C*/IC,** 0,6 ml;
- **PCR-mix-1-FL *Sabin* 1/2/3,** 0,6 ml;
- **PCR-mix-2-FRT,** 2 x 0,3 ml;
- **TaqF Polymerase**, 2 x 0,03 ml;
- **Internal Control (IC RNA)***, 5 x 0,12 ml.
- **Negative Control C-****, 1,2 ml;
- **Pos IC DNA**, 0,6 ml.
- **Positive Control cDNA *HEV-C* C+,** 2 x 0,1 ml;
- **Positive Control cDNA *Sabin* 1/2/3 C+,** 2 x 0,1 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 reactions

* *must be used in the extraction procedure as Internal Control (IC).*
** *must be used in the extraction procedure as Negative control of extraction.*

Module No.2: Complete Real Time PCR test with RNA purification kit (TV58-50FRT)

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 – **"Reverta-L":** Reverse transcription of the RNA

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 3 – **"Poliovirus Real-TM":** Real Time amplification kit

- **PCR-mix-1 *HEV-C*/IC,** 0,6 ml;
- **PCR-mix-1-FL *Sabin* 1/2/3,** 0,6 ml;
- **PCR-mix-2-FRT,** 2 x 0,3 ml;
- **TaqF Polymerase**, 2 x 0,03 ml;
- **Internal Control (IC RNA)***, 5 x 0,12 ml.
- **Negative Control C-****, 1,2 ml;
- **Pos IC DNA**, 0,6 ml.
- **Positive Control cDNA *HEV-C* C+,** 2 x 0,1 ml;
- **Positive Control cDNA *Sabin* 1/2/3 C+,** 2 x 0,1 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 reactions

* *must be used in the extraction procedure as Internal Control (IC).*
** *must be used in the extraction procedure as Negative control of extraction.*

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 ± 2°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 Thermalcycler
- Workstation
- Pipettors
- Sterile tips with filters
- Tube racks

STORAGE INSTRUCTIONS

Kits can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -20°C immediately on receipt. **Reverta-L** and **Poliovirus Real-TM** must be stored at -20°C. **Ribo-Sorb** must be stored at 2-8°C.

STABILITY

Poliovirus 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. 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

Poliovirus Real-TM can analyze RNA extracted from:

- *water:* centrifuge 10-20 ml for 10 min at maximum speed. Discard the supernatant and leave about 100 µl of solution for RNA extraction;
- *Cerebrospinal fluid;*
- *feces:*

  Transfer 0.4-1.0 g (≤ 1 ml) of feces to a sterile vial using sterile spatula. Add 4.0 ml of saline solution to obtain 10-20 % suspension. Mix the vial on the vortex. Decolorize the suspension by centrifuging for 20 min at 3000 rmp. Use supernatant for RNA extraction. Transfer extract to a sterile tube for storing.

  N.B. Liquid feces can be used without suspension preparing stage.

  Store fecal extract for 1 day at 2-8 °C, for 1 month (with addition of glycerol) at ≤ –16 °C, for a long time (with addition of glycerol) at ≤ –68 °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);

⇒ **SaMag Viral Nucleic Acid Extraction kit** (Sacace, REF SM003).

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

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 IC**.
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 **C– 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.

RT AND AMPLIFICATION

Reverse Transcription:

1) Prepare Reaction Mix: for 12 reactions, **add 5,0 µl RT-G-mix-1** into the tube containing **RT-mix** and vortex for at least 5-10 seconds, centrifuge briefly. This mix is stable for 1 month at -20°C. Add **6 µl M-MLV** into the tube with Reagent Mix, mix by pipetting, vortex for 3 sec, centrifuge for 5-7 sec (must be used immediately after the preparation).

   *(If it is necessary to test less than 12 samples add for each sample (N) in the new sterile tube **10*N µl** of **RT-G-mix-1 with RT-mix** and **0,5*N µl** of **M-MLV**).*

2) Add **10 µl** of **Reaction Mix** into each sample tube.
3) Pipette **10 µl RNA** samples to the appropriate tube. (*If the Ribo-Sorb isolation kit is used as a RNA extraction kit, re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction)*.Carefully mix by pipetting.
4) Place tubes into thermalcycler and incubate at 37°C for 30 minutes.
5) Dilute 1: 2 each obtained cDNA sample with TE-buffer (add **20 µl TE-buffer** to each tube).

cDNA specimens could be stored at -20°C for a week or at -70°C during a year.

PCR Reagents preparation

The total reaction volume is **25 µl**, the volume of cDNA sample is **10 µl**.

1. Thaw the reagents and vortex the tubes thoroughly.
2. Prepare the required number of tubes including controls.
3. Mix **PCR-mix-1-FL *HEV-C*/STI** with **PCR-mix-2-FRT** and **TaqF polymerase** and **PCR-mix-1-FL *Sabin* 1/2/3** with **PCR-mix-2-FRT** and **polymerase (TaqF)** according to **Table 1**. Vortex the tubes thoroughly.

Table 1. **Preparation of reaction mixture**

Reagent volume for 1 reaction (µl)	**10.00**	**5.00**	**0.50**
The quantity of reactions *	**PCR-mix-1-FL**	**PCR-mix-2-FRT**	**Polymerase (TaqF)**
8	**80**	**40**	**4.0**
10	**100**	**50**	**5.0**
12	**120**	**60**	**6.0**
14	**140**	**70**	**7.0**
16	**160**	**80**	**8.0**
18	**180**	**90**	**9.0**
20	**200**	**100**	**10.0**
22	**220**	**110**	**11.0**
24	**240**	**120**	**12.0**
26	**260**	**130**	**13.0**
28	**280**	**140**	**14.0**
30	**300**	**150**	**15.0**
32	**320**	**160**	**16.0**

* for **PCR-mix-1 *HEV-C*/IC**: the quantity of samples + neg extr. control + 2 controls of amplification + one extra sample (N+3+1);

* for **PCR-mix-1 *Sabin* 1/2/3:** the quantity of samples + neg extr. control + 1 control of amplification + one extra sample (N+2+1).

4. Transfer **15 µl** of the prepared mixture to the prepared tubes.
5. Add **10 µl** of **cDNA** obtained from clinical or control samples at the reverse transcription stage into the prepared tubes using tips with aerosol barrier.
6. Carry out the control amplification reactions:

NCA - Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+ - Add **10 µl** of **Positive Control cDNA *HEV-C*** (for PCR-mix-1-FL *HEV-C*/IC) and **10 µl** of **Positive Control cDNA *Sabin* 1/2/3** (for PCR-mix-1-FL *Sabin* 1/2/3) to the tube labeled C+ (Positive Control of Amplification).

CS+ - Add 10 µl of **Pos IC DNA** (for PCR-mix-1 *HEV-C*/IC) to the tube labeled CS+ (Positive Control of Amplification of IC).

Amplification

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

	Rotor type Instruments[1]			**Plate or Module type Instruments**[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
Hold	**95**	15 min	1	**95**	15 min	1
Cycling	**95**	10 s	45	**95**	10 s	45
	54	20 s *fluorescent signal detection*		**54**	30 s *fluorescent signal detection*	
	72	10 s		**72**	10 s	

[1] *For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)*
[2] *For example,* SaCycler-96™ *(Sacace), iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)*

RESULTS ANALYSIS

The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line. Fluorescent signal is detected in the channels designed for the FAM/Green, JOE/Yellow/HEX/Cy3 and ROX/Orange/TexasRed fluorophores on the 2nd step of stage Cycling.

PCR-mix-1 *HEV-C*/IC:

IC is detected in the JOE/Yellow/HEX/Cy3 fluorescence channel;

HEV-C cDNA is detected in the FAM/Green fluorescence channel.

PCR-mix-1 *Sabin* 1/2/3:

Sabin 1 cDNA is detected in the ROX/Orange/TexasRed fluorescence channel;

Sabin 2 cDNA is detected in the FAM/Green fluorescence channel;

Sabin 3 cDNA is detected in the JOE/Yellow/HEX/Cy3 fluorescence channel.

Interpretation of results for PCR-mix-1 *HEV-C*/IC:

Ct value in channel		Interpretation
FAM/Green	**JOE/Yellow/HEX/Cy3**	
Pos	Pos	***HEV-C*** cDNA is **detected**
Pos	Neg	***HEV-C*** cDNA is **not detected**
Neg	Neg	**Invalid result**

Interpretation of results for PCR-mix-1-FL *Sabin* 1/2/3:

Ct value in channel			Interpretation
FAM/Green	**JOE/Yellow/HEX**	**ROX/Orange**	
Pos	Neg	Neg	*Sabin* 2 cDNA is **detected**
Neg	Pos	Neg	*Sabin* 3 cDNA is **detected**
Neg	Neg	Pos	*Sabin* 1 cDNA is **detected**
Neg	Neg	Neg	*Sabin* 1/2/3 cDNA are **not detected****

** If the result is positive for PCR-mix-1 HEV-C/IC in the FAM/Green channel.

Results for controls:

PCR-mix-1	Control	Stage for control	Ct value in channel		
			FAM/Green	**JOE/Yellow/HEX/ Cy3**	**ROX/Orange**
***HEV-C*/IC**	C–	RNA extraction	Pos	Neg	–
***HEV-C*/IC**	C+	Amplification	Neg	Pos	–
***HEV-C*/IC**	CS+	Amplification	Pos	Neg	–
***HEV-C*/IC**	NCA	Amplification	Neg	Neg	–
***Sabin* 1/2/3**	C–	RNA extraction	Neg	Neg	Neg
***Sabin* 1/2/3**	C+	Amplification	Pos	Pos	Pos
***Sabin* 1/2/3**	NCA	Amplification	Neg	Neg	Neg

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **Poliovirus Real-TM** kit is ensured by selecting specific primers and probes as well as by strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks. The clinical specificity of **Poliovirus Real-TM** PCR kit was confirmed in laboratory clinical trials.

Specificity was checked while testing DNA samples of following microorganisms: *Enterovirus* (*Coxsakie* B1, B2, B3, B4, B5, B6; *Polio (Sabin)* I, II, III); *Influenza virus* A (H13N2, H9N2, H8N4, H2N3, H4N6, H11N6, H12N5, H3N8, H1N1, H6N2, H10N7, H5N1), *Influenza virus B*, *Rhinovirus*, *RS virus*, human *Adenovirus* – 3, 5, 7, 37, 40.

Specificity was estimated while testing DNA samples of following microorganisms: *N.meningitides, St pneumoniae, H.influenzae, Clebsiella* K 65 SW4, *Listeria monocytogenes* USHC 19, *Listeria monocytogenes* USHC 52, *Proteus vulgaris* 115/98, *Pseudomonas aeruginosa* DN c1, *Staphylococcus aureus* 653, *Staphylococcus aureus* 29112, *Morganella Morganii* 619 c 01, *Enterobacter faecalis* 356.

Analytical sensitivity

The analytical sensitivity of the kit **Poliovirus Real-TM** is not less than 1000 copies/ml.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel) with PCR-mix-1 HEV-C/IC : 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.
        - ⇒ If the Ribo-Sorb isolation kit is used as a RNA extraction kit, re-centrifuge all the tubes before pipetting the extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - 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. Joe (Yellow)/Cy3/HEX signal 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.
3. Any signal with Negative PCR Control (NCA).
    - 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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