IVD For *in Vitro* Diagnostic Use

CE

Rotavirus/Norovirus/Astrovirus Real-TM

Handbook

Real Time PCR kit for qualitative detection of *Rotavirus A, Norovirus 2 genotype, Astrovirus*

REF V40-50FRT

REF TV40-50FRT

Σ **50**

NAME

Rotavirus/Norovirus/Astrovirus Real-TM

INTRODUCTION

Acute Intestinal Infection (A.I.I.) are one of the primary causes of hospitalization in infectious disease departments. In accordance with the data provided by the contemporary literature, the most often detectable and generally spread etiological agents of A.I.I. are bacterial microorganisms such as *Shigella spp*. and enteroinvasive *E. coli* (EIEC), *Salmonella spp*., thermophilic group of Campylobacter spp., enteropathogenic E.coli (EPEC) and enteroaggregative E. coli (EAEC) and viral agents such as group A rotaviruses, genotype 2 noroviruses, group F adenoviruses (type 40 and 41) and astroviruses.

INTENDED USE

Kit **Rotavirus/Norovirus/Astrovirus Real-TM** is a Real-Time test for the qualitative detection and differentiation of *Rotavirus A, Norovirus 2 genotype, Astrovirus* in the biological materials and in the environment. RNA is extracted from specimens, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for Rotavirus/Norovirus/Astrovirus RNA and IC (Internal Control).

PRINCIPLE OF ASSAY

Rotavirus/Norovirus/Astrovirus Real-TM Test is based on three major processes: isolation of *virus* RNA from specimens, one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *Rotavirus/Norovirus/Astrovirus* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **Rotavirus/Norovirus/Astrovirus Real-TM** PCR kit is a qualitative test which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V40-50FRT)

Part N° 2 – **"Rotavirus/Norovirus/Astrovirus Real-TM":** Real Time amplification kit

- **PCR-mix-1 *Rotavirus* / *Astrovirus***, 0,6 ml;
- **PCR-mix-1 *Norovirus* / *IC*** 0,6 ml;
- **RT-PCR-mix-2**, 2 x 0,3 ml;
- **Hot Start Taq Polymerase**, 2 x 0,03 ml;
- **M-MLV Revertase**, 2 x 0,015 ml;
- **RT-G-mix-2**, 2 x 0,015 ml;

Contains reagents for 55 reactions

Part N° 3 – **"Controls"**

- **Negative Control C-**, 1,6 ml;*
- **Internal Control (IC RNA)**, 5 x 0,12 ml.**
- **Pos cDNA *Rotavirus*/*Astrovirus* C+,** 0,1 ml;
- **Pos cDNA *Norovirus* 2 / *Internal Control (IC)* C+,** 0,1 ml;
- **DNA-buffer**, 0,5 ml;

** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg*
***add 10 µl of Internal Control to all samples during the RNA isolation procedure directly to the sample/lysis mixture*

Module No.2: Complete Real Time PCR test with RNA purification kit (TV40-50FRT)

Part N° 1 – **“Ribo-Sorb”:**

- **Lysis Solution**, 22,5 ml;
- **Washing Solution**, 20 ml;
- **Sorbent**, 1,25 ml.
- **RNA-eluent**, 5 x 1,2 ml;

Contains reagents for 50 tests.

Part N° 2 – **“Rotavirus/Norovirus/Astrovirus Real-TM”:**

- **PCR-mix-1 *Rotavirus* / *Astrovirus***, 0,6 ml;
- **PCR-mix-1 *Norovirus* / *IC*** 0,6 ml;
- **RT-PCR-mix-2**, 2 x 0,3 ml;
- **Hot Start Taq Polymerase**, 2 x 0,03 ml;
- **M-MLV Revertase**, 2 x 0,015 ml;
- **RT-G-mix-2**, 2 x 0,015 ml;

Contains reagents for 55 reactions

Part N° 3 – **“Controls”**

- **Negative Control C-**, 1,6 ml;*
- **Internal Control (IC RNA)**, 5 x 0,12 ml;**
- **Pos cDNA *Rotavirus*/*Astrovirus* C+,** 0,1 ml;
- **Pos cDNA *Norovirus* 2 / *Internal Control (IC)* C+,** 0,1 ml;
- **DNA-buffer**, 0,5 ml;

** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg*
***add 10 µl of Internal Control to all samples during the RNA isolation procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation (only for Module No. 2):

- 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 (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

STORAGE INSTRUCTIONS

Ribo-Sorb kit must be stored at 2-8°C; **Rotavirus/Norovirus/Astrovirus Real-TM”** must be stored at -20°C; **Controls** must be stored at 2-8°C.

The 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.

STABILITY

Rotavirus/Norovirus/Astrovirus 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

Rotavirus/Norovirus/Astrovirus 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 DNA extraction;
- *whole blood* collected in EDTA tubes*;*
- *feces:*
  - Prepare 20% feces suspension by adding in 5 ml tube of 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g and using a micropipette with a plugged aerosol barrier tip transfer in a new sterile 1,5 ml tube 50 µl of the bacterial fraction (white-yellowish line between the sediment and the supernatant)

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.

DNA 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).

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 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 **50 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 al 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.

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

RT AND AMPLIFICATION

Total reaction volume is **25 µl**, the volume of RNA sample is **10 µl**.

1 Prepare required quantity of reaction tubes (2 tubes for each sample + Controls)

2 Prepare the reaction mix for required number of samples.

3 For N reactions mix for each PCR-Mix-1 in a new tube:

10*(N+1) µl of RT-PCR-mix-1 *Rotavirus* / *Astrovirus* (or *Norovirus* / *IC)*

5.0*(N+1) µl of RT-PCR-mix-2

0.5*(N+1) µl of Polymerase

0.25*(N+1) µl of RT-G-mix-2

0.25*(N+1) µl of MMlv

4 Vortex the tube, then centrifuge shortly. Add **15 µl** of prepared reaction mix into each appropriate tube.

5 Using tips with aerosol filter add **10 µl** of RNA samples obtained at the stage of RNA isolation and mix carefully by pipetting**.**

N.B. 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

6 Prepare for each panel 3 controls:

- add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
- add **10 µl** of **Pos cDNA *Rotavirus*/*Astrovirus*** to the tube with **PCR-mix-1 *Rotavirus* / *Astrovirus;***
- add **10 µl** of **Pos cDNA *Norovirus* 2 / *IC* C+** to the tube with **PCR-mix- *Norovirus* / IC**

Amplification

Create a temperature profile on your Real-time instrument as follows:

	***Rotor type instruments*[1]**				***Plate type or modular instruments*[2]**			
Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp,°C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	50	30 min	–	1	50	30 min	–	1
Hold	95	15 min	–	1	95	15 min	–	1
Cycling 2	95	10 s	–	45	95	10 s	–	45
	60	25 s	FAM(Green), JOE(Yellow)		60	35 s	FAM, JOE/HEX/Cy3	
	72	10 s	–		72	10 s	–	

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*

[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.05*	*5-10 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.05*	*10 %*	*On*

Plate-type instruments

For each channel set the threshold line at the level of 10-20 % of HEX maximum fluorescence obtained for the C+ in the last amplification cycle.

Boundary Ct values

All types of test material

RT-PCR-mix-1-FEP/FRT	Ct value	
	FAM/Green	JOE/Yellow/HEX
RT-PCR-mix-1-FEP/FRT *Rotavirus* / *Astrovirus*	**40**	**40**
RT-PCR-mix-1-FEP/FRT *Norovirus* / STI	**40**	**40**

RESULTS ANALYSIS

1. The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.
   - Internal Control (IC) is detected on the FAM (Green) channel and *Norovirus* on the JOE (Yellow)/HEX/Cy3 channel with PCR-mix- *Norovirus* / IC;
   - *Rotavirus A* is detected on the FAM (Green) channel and *Astrovirus* on the JOE (Yellow)/HEX/Cy3 channel with PCR-mix-1 *Rotavirus* / *Astrovirus;*
2. The sample is considered to be positive if the value of **Ct** is different from zero and lower than 33 (Ct<40 for enviromental samples).
3. The sample is considered to be negative if the result is positive only on the channel Fam with PCR-mix- *Norovirus* / IC STI-87-rec and the Ct value is lower than 33 (Ct<40 for enviromental samples).

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **Rotavirus/Norovirus/Astrovirus Real-TM** PCR kit is specified in the table below.

Pathogen	Test material	RNA/DNA extraction kit	Analytical sensitivity, GE/ml*
Rotavirus A	Feces	DNA/RNA Prep	1×10^3
Norovirus genotype 2	Feces	DNA/RNA Prep	5×10^2
Astrovirus	Feces	DNA/RNA Prep	1×10^3

** Genome equivalents (GE) of the microorganism per 1 ml of a sample.*

Specificity

The analytical specificity of **Rotavirus/Norovirus/Astrovirus Real-TM** PCR kit is ensured by selection of specific primers and probes as well as strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Specificity was confirmed on the following microorganism strains:

GISK collection: *Enterovirus* strains (Coxsakie B1, B2, B3, B4, B5, and B6; Polio (Sabin) I, II, and III). *Adenovirus* serogroups 5 and 7; *influenza virus* A (H13N2, H9N2, H8N4, H2N3, H4N6, H11N6, H12N5, H3N8, H1N1, H6N2, H10N7, and H5N1) and B; *rhinovirus*; *RS viruses*; and human *adenovirus* types 3, 5, 7, 37, and 40.

VGNKI collection: *Salmonella enteritidis* S-6, *S.choleraesuis* 370, *S.typhimurium* 371, *S.dublin* 373, *S.typhi* C1, *S.abortusovis 372*, and *S.gallinarum-pullorum;*, *Shigella flexneri* 851b; *Campylobacter fetus* ssp. *fetus* 25936 and *C.jejuni* ssp. *jejuni* 43435; *Clebsiella* K 65 SW4; *Listeria monocitogenes* USKHCH 19 and *L.monocitogenes* USKHCH 52; *Proteus vulgaris* 115/98; *Pseudomonas aeruginosa* DN c1; *Staphilococcus aureus* 653 and *S. aureus* 29112; *Morganella morganii* 619 c 01; and *Enterobacter faecalis* 356.

Center for Disease Control and Prevention (CDC, USA) collection: 44 isolates of *norovirus* genotype 1 and 2 different genetic clusters; 40 strains of different *rotavirus* [P]G types, 19 strains of *astrovirus* serotypes 1, 2, 4, 5, and 8; and 15 strains of different *adenovirus* types and the following bacterial strains (see table below).

Table. **The panel of bacterial pathogens Center for Disease Control and Prevention (CDC, USA)**

Strain ID	Organism	Strain ID	Organism
K2033	*Salmonella* ser. Grumpensis	K2015	*Salmonella* ser. Oranienburg
K1806	*Salmonella* ser. Newport	AM01144	*Salmonella* ser. Newport
K2077	*Salmonella* ser. Enteriditis	K1810	*Salmonella* ser. Anatum
83-99	*Salmonella* ser. Typhimurium	K1991	*Salmonella* ser. Typhimurium
PS505	*Shigella boydii*	K1898	*Salmonella* ser. Heidelberg
PS408	*Shigella sonnei*	PS555	*Shigella boydii*
B4003	*Shigella sonnei*	F6446	*Shigella dysenteriae*
PS801	*Shigella dysenteriae*	S821X1	*Shigella dysenteriae* type 1
C898	*Shigella dysenteriae* type1	S177X1	*Shigella dysenteriae* type 1
F2035	*Shigella flexneri*	S3314	*Shigella dysenteriae* type 2
E2539-C1	Enterotoxigenic *Escherichia coli* (ETEC)	PS071	*Shigella flexneri*
H10407	Enterotoxigenic *Escherichia coli* (ETEC)	PS050	*Shigella flexneri*
F1008	Enterotoxigenic *Escherichia coli* (ETEC)	F7862	*Shigella flexneri*
EDL 933	Shiga-toxin *E. coli* (STEC)	TX1	Enterotoxigenic *Escherichia coli* (ETEC)
3543-01	Shiga-toxin *E. coli* (STEC)	3525-01	Shiga-toxin *Escherichia coli* (STEC)
4752-71	*Proteus vulgaris*	25922	*Escherichia coli* O6:H1
QA/QC	*Citrobacter freundii*	621-64	*Citrobacter freundii*
QA/QC	*Aeromonas*	3910-68	*Aeromonas spp.*
3043-74	*Serratia marcescens*	E9113	*Vibrio cholerae*
QA/QC	*Serratia marcescens*	501-83	*Edwardsiella spp.*
F7894	*Vibrio vulnificus*	587-82	*Providencia stuartii*
F8515	*Yersinia enterocolitica*	27853	*Pseudomonas aeruginosa*
F8510	*Yersinia enterocolitica*	D4989	*Helicobacter cineadi*
K4299	*Vibrio parahaemolyticus*	D6827	*Helicobacter pullorum*
F9835	*Vibrio parahaemolyticus*	D5127	*Helicobacter pylori*
K2023	*Salmonella* ser. Kentucky	D2686	*Arcobacter butzleri*
K1684	*Salmonella* O-1, 4, 12 gr. B		

There were no nonspecific test responses during examination of human DNA as well as a DNA panel of the above-mentioned microorganisms.

TROUBLESHOOTING

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

- If the signal for C– (except for C– in JOE/Yellow/HEX channel for RT-PCR-mix-1-FEP/FRT Norovirus / STI) and/or the signal for NCA in the JOE/Yellow/HEX and/or FAM/Green channel is less than the boundary value, analysis should be repeated starting from the RNA extraction stage.
- If no signal is detected for the positive controls of amplification, it may suggest that the programming of the temperature profile of the used Instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.
- If a positive result (the fluorescence curve crosses the threshold line) is detected for a sample that has a fluorescence curve without the typical exponential growth phase (the curve is linear), this may suggest incorrect setting of the threshold line or incorrect calculation of baseline parameters. Such a result should not be considered as positive.

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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