Instruction for Use

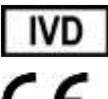
virellaNoro

real time RT-PCR Kit TM

For qualitative *in vitro* detection of Norovirus RNA (genotype I and genotype II) in stool samples as well as in food and environmental samples.

REF	G01008-32	G01008-96
Σ	32	96

-18°C



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Index

1 Intended Use 3
2 Pathogen Information 3
3 Principle of the Test 3
4 Package Contents 4
5 Equipment and Reagents to be Supplied by User 4
6 Transport, Storage and Stability 4
7 Important Notes 5
8 General Precautions 5
9 Sample Material 5
10 Sample Preparation 5
11 Control RNA 6
12 Real time RT-PCR 7
12.1 Important Points Before Starting: 7
12.2 Procedure 7
12.3 Instrument Settings 9
13 Data Analysis 10
14 Assay Validation 12
15 Limitations of the Method 12
16 Troubleshooting 13
17 Kit Performance 14
17.1 Diagnostic Sensitivity and Specificity 14
17.2 Analytical Sensitivity 15
18 Abbreviations and Symbols 17
19 Literature 17

1 Intended Use

The virellaNoro real time RT-PCR TM is an assay for the detection of Norovirus RNA (Genogroup I and II) in stool, food and environmental samples using real time PCR microplate systems.

2 Pathogen Information

Noroviruses are small non-enveloped RNA viruses belonging to the family of Caliciviridae. They cause approximately 90 % of epidemic non-bacterial outbreaks of gastroenteritis around the world.
The viruses are transmitted by fecally contaminated food or water and by person-to-person contact. For this reason, outbreaks of Norovirus infection often occur in closed or semi-closed communities, such as long-term care facilities, hospitals, prisons, dormitories, and cruise ships.
Noroviruses are highly contagious and are stable at temperatures between -20°C to +60°C and in acidic environments up to pH 3.
Norovirus infections occur throughout the year, however, in Europe, seasonal increases are observed between October and March.
The virellaNoro real time RT-PCR Kit TM detects Norovirus strains of high genetic diversity, such as the following:
GI: Norwalk, Desert Shield, Winchester, Queensarms, Southhampton, Chiba
GII: Lordsdale, Bristol, Melksham, Toronto, Hawaii

3 Principle of the Test

The virellaNoro real time RT-PCR Kit TM contains specific primers and dual-labeled probes for the amplification and detection of Norovirus RNA (GI and GII) in stool, food and environmental samples after the extraction of RNA from the sample material.
The reverse transcription (RT) of viral RNA to cDNA and the subsequent amplification of Norovirus specific fragments are performed in a one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the Polymerase. The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.
The fluorescence of the pathogen-specific probes is measured in the FAM channel.
Furthermore, virellaNoro real time RT-PCR Kit TM contains a Control RNA, which is added during RNA extraction and detected in the same reaction by a differently labeled probe.

The Control RNA allows the detection of RT-PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen.
The fluorescence of the Control RNA is measured in the VIC®/HEX/JOE/TET channel.

4 Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the virellaNoro real time RT-PCR Kit TM.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
Enzyme	blue	1 x 6.4 µl	1 x 19.2 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control RNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortex mixer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: VLP-RNA (Virus-Like Particles, please look at page 6 for details).

6 Transport, Storage and Stability

The virellaNoro real time RT-PCR Kit is shipped on dry ice or cool packs. All components must be stored at maximum -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.
Up to 20 freeze and thaw cycles are possible.

For convenience, opened reagents can be stored at +2-8°C for up to 6 months. Protect kit components from direct sunlight during the complete test run.

7 Important Notes

- The virellaNoro real time RT-PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the RT-PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine virellaNoro real time RT-PCR Kit components of different lot numbers.

9 Sample Material

Starting material for the virellaNoro real time RT-PCR is viral RNA isolated from clinical specimens (stool, food, or environmental samples).

10 Sample Preparation

The virellaNoro real time RT-PCR is suitable for the detection of Norovirus RNA isolated from clinical specimens with appropriate isolation methods.
Commercial kits for RNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

Stool samples:

Suspend some stool sample (approx. the size of a pea) in 1.5 ml sterile dH_2O. Vortex well. Once the solid components have sunken to the bottom of the tube (where necessary centrifuge for 5 min at 3.500 x g) use the supernatant for extraction.

Very liquid stool samples can be used for extraction without further suspension in water. Also vortex well and wait for the solid components to sink to the bottom of the tube (where necessary centrifuge for 5 min at 3500 g).

Important: In addition to the samples always run a ‚water control' in your extraction. Treat this water control analogous to a sample.
Comparing the amplification of the Control RNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note the chapter 11 ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the RNA extraction kit's manufacturer.

11 Control RNA

A Control RNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.
The Virus-Like Particles (VLP-RNA) are not supplied.

RNA isolation from stool, food, or environmental samples

a) Control RNA or VLP-RNA used as Extraction Control:

virellaNoro Control RNA or VLP-RNA is added to the RNA extraction.
Add 5 µl Control RNA or VLP-RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions. Please follow protocol A.

The Control RNA must be added to the Lysis Buffer of the extraction kit.

b) Control RNA used as Internal Control of the real time RT-PCR:

If only inhibition will be checked please follow protocol B.

12 Real time RT-PCR

12.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Important Notes'.
- Before setting up the real time RT-PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the RT-PCR set up.
- In every RT-PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents - except the Enzyme - should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

12.2 Procedure

If the Control RNA or VLP-RNA is used to control both, the real time RT-PCR and the RNA isolation procedure, please follow protocol A. If the Control RNA is solely used to detect possible inhibition of the real time RT-PCR, please follow protocol B.

Protocol A

The Control RNA or VLP-RNA was added during RNA extraction (see chapter 11 ‚Control RNA'). In this case, prepare the Master Mix according to Table 2.

The Master Mix contains all of the components needed for RT-PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix (Control RNA was added during RNA extraction)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Protocol B

The Control RNA is used for the control of the real time RT-PCR only (see chapter 11, Control RNA‘). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real RT-PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the Master Mix (Control RNA is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)
0.2 µl Control RNA*	0.2 µl x (N+1)*

*The increase in volume caused by adding the Control RNA is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time RT-PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the RNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time RT-PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time RT-PCR use the thermal profile shown in Table 5.

Table 5: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification			
Denaturation	10 sec	95°C	45
Annealing	40 sec Aquisition at the end of this step	60°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to Table 6.

Table 6: Overview of the instrument settings required for the virellaNoro real time RT-PCR TM.

Real time RT-PCR Instrument	Parameter	Detection Channel	Notes	
LightCycler 480I	Norovirus	483-533	pre-installed universal CC FAM (510) – VIC (580)	
	Control RNA	523-568		
LightCycler 480II	Norovirus	FAM (465-510)		
	Control RNA	HEX (533-580)		
Stratagene Mx3000P / Mx3005P	Norovirus	FAM	Gain 8	Reference Dye: None
	Control RNA	HEX	Gain 1	
ABI 7500	Norovirus	FAM	Option Reference Dye ROX: NO	
	Control RNA	JOE		
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Norovirus	Green	Gain 5	
	Control RNA	Yellow	Gain 5	

13 Data Analysis

The Norovirus specific amplification is measured in the FAM channel. The amplification of the Control RNA is measured in the VIC®/HEX/JOE/TET channel.

Following results can occur:

- A signal in the FAM channel is detected:
  The result is positive, the sample contains Norovirus RNA.
  In this case, detection of a signal of the Control RNA in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the Control RNA.
- No signal in the FAM channel, but a signal in the VIC®/HEX/JOE™/TET channel is detected:
  The result is negative, the sample does not contain Norovirus RNA.
  The signal of the Control RNA excludes the possibilities of RNA isolation failure (in case the Control RNA is being used as an Extraction Control) and/or real time RT-PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“).
- Neither in the FAM nor in the VIC®/HEX/JOE™/TET channel a signal is detected:
  A diagnostic statement cannot be made.
  The RNA isolation was not successful or an inhibition of the RT-PCR has occurred. In case the Control RNA was added during RNA isolation and not directly to the PCR Master Mix, the Negative Control is negative in both channels.

Figure 1 and **Figure 2** show examples for positive and negative real time RT-PCR results.

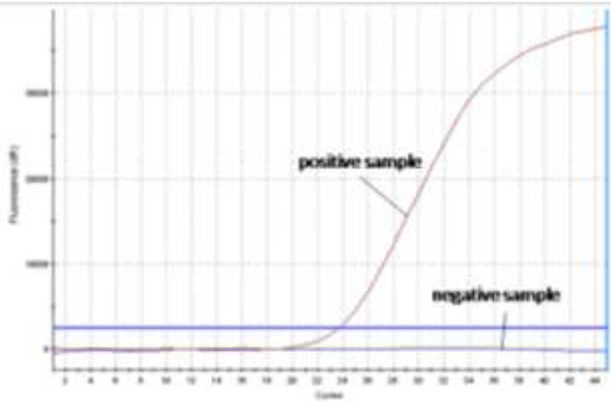

Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

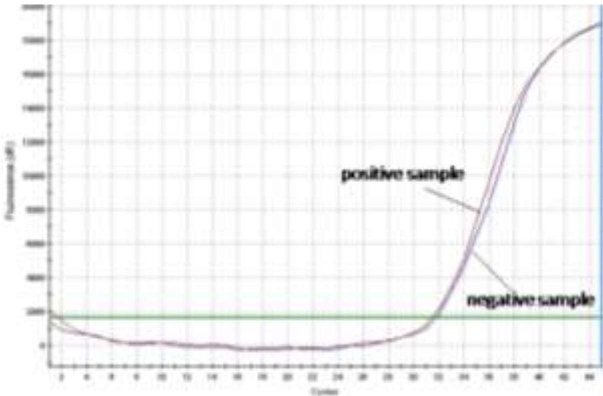

Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA-specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus-specific FAM channel is not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative.

14 Assay Validation

Set a threshold as follows:

Negative Controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 33.

15 Limitations of the Method

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude a Norovirus infection.

16 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR.

No fluorescence signal in the FAM channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the virus specific amplification and the VIC®/HEX/JOETM/TET channel for the amplification of the Control RNA.
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with ‚Procedure' on page 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 9).
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 4.
Weak or no signal of the Control RNA and simultaneous absence of a signal in the virus specific FAM channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 7).
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the RNA.
RNA loss during isolation process	In case the Control RNA was added before extraction, the lack of an amplification signal can indicate that the RNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 4.

Detection of a fluorescence signal in the FAM channel of the Negative Control	
Contamination during preparation of the RT-PCR	Repeat the real time RT-PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time RT-PCR.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the virellaNoro real time RT-PCR Kit TM 45 positive and 50 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 % (tables 7 and 8). Furthermore, in silico analysis of primers and probes showed no cross-reactivities to sequences of other gastroenteric pathogens.
The positive predictive value was found to be 100%, the negative predictive value showed to be 100%.

Table 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
virellaNoro positive	45	0
virellaNoro negative	0	50

Sensitivity	100 %
Specificity	100 %

Table 8: Diagnostic Specificity

Virus/Bacterium	Number of strains tested	Result
Norovirus	45	positive
Enterovirus	11	negative
Adenovirus	1	negative
Rotavirus	1	negative
Astrovirus	1	negative
Sapovirus	1	negative
Listeria monocytogenes	1	negative
Campylobacter	1	negative
Citrobacter	1	negative
Salmonella	1	negative
E. coli	1	negative

17.2 Analytical Sensitivity

The Analytical Sensitivity virellaNoro real time RT-PCR Kit TM was determined using round robin samples of QCMD ring trials. Table 9 shows the results of the tests. All of the positive samples could be detected even in the highest dilutions.

Table 9: Results of testing of QCMD Norovirus Panels 2008-2011 with virellaNoro real time RT-PCR Kit TM.

Panel Code	Matrix	Strain	Dilution Factor	Result virellaNoro real time RT-PCR TM
NV08-01	VTM	*GII.4*	$1x10^{-3}$	positive
NV08-02	VTM	*GI.3*	$1x10^{-4}$	positive
NV08-03	VTM	*GII.4*	$5x10^{-3}$	positive
NV08-04	VTM	*GI.3*	$5x10^{-3}$	positive
NV08-05	VTM	*GII.4*	$1x10^{-2}$	positive
NV08-06	VTM	*GI.3*	$1x10^{-2}$	positive
NV08-07	VTM	*GI.3*	$1x10^{-3}$	positive
NV08-08	VTM	negative		negative
NV08-09	VTM	*GII.4*	nn	positive
NV08-10	TE-Puffer	*GIV.1*	nn	positive
NV08-11	TE-Puffer	*GII.4*	nn	positive
NV08-12	TE-Puffer	negative	nn	negative
NV08-13	TE-Puffer	*GI.7*	nn	positive
NV08-14	TE-Puffer	*GI.3*	nn	positive
NV09-01	VTM	*GII.4*	$1x10^{-5}$	positive
NV09-02	VTM	*GI.3*	$1x10^{-2}$	positive
NV09-03	VTM	*GII.4*	$1x10^{-2}$	positive
NV09-04	VTM	negative		negative
NV09-05	VTM	*GI.3*	$1x10^{-3}$	positive
NV09-06	VTM	*GII.4*	$1x10^{-4}$	positive
NV09-07	VTM	*GII.4*	$1x10^{-3}$	positive
NV09-08	TE-Puffer	*GI.7*	$1x10^{-3}$	positive
NV09-09	TE-Puffer	negative		negative
NV09-10	TE-Puffer	*GI.3*	$1x10^{-3}$	positive
NV09-11	TE-Puffer	*GII.4*	$1x10^{-3}$	positive
NV09-12	TE-Puffer	*GIV.1*	$1x10^{-3}$	positive
NV10-01	VTM	*GII.4*	$1x10^{-4}$	positive
NV10-02	VTM	*GII.4*	$1x10^{-2}$	positive
NV10-03	VTM	*GI.3*	$1x10^{-2}$	positive
NV10-04	VTM	negative		negative
NV10-05	VTM	*GI.3*	$1x10^{-1}$	positive
NV10-06	VTM	*GII.4*	$1x10^{-1}$	positive
NV10-07	VTM	*GII.4*	$1x10^{-3}$	positive
NV10-08	TE-Puffer	*GI.3*	$1x10^{-3}$	positive
NV10-09	TE-Puffer	*GIV.1*	$1x10^{-3}$	positive
NV10-10	TE-Puffer	*GI.7*	$1x10^{-3}$	positive

18 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid	REF	Catalog number
RNA	Ribonucleid Acid	Σ	Contains sufficient for <n> test
PCR	Polymerase Chain Reaction	-18°C	Upper limit of temperature
RT	Reverse Transcription		Manufacturer
nn	not known		Use by YYYY-MM
VTM	Virus Transport Medium	LOT	Batch code
REACTION MIX	Reaction Mix	CONT	Content
ENZYME	Enzyme	i	Consult instructions for use
CONTROL +	Positive Control	IVD	*In vitro* diagnostic medical device
CONTROL –	Negative Control	CE	European Conformity
CONTROL RNA IC	Control RNA		

19 Literature

[1] M. K. Koopmans et al.: *Genus Norovirus*. In: C.M. Fauquet, M.A. Mayo et al.: *Virus taxonomy: classification and nomenclature of viruses: eighth report of the International Committee on the Taxonomy of Viruses.* Academic Press, London/ San Diego 2005, S. 847f.

[2] Robert-Koch-Institut, RKI. Noroviren. ww.rki.de/DE/Content/InfAZ/N/Noroviren/Noroviren.html