Instructions for Use

In-vitro-Diagnostic

The Norovirus *direct* RT-PCR is a real-time RT-PCR test for in vitro detection of RNA from norovirus (GI and GII).

The Norovirus *direct* RT-PCR test was validated with the Roche LightCycler® 480 II and with the BioRad CFX Opus 96™. In general, *direct* tests are compatible with other qPCR cycler (e.g., MIC Cycler, AJ qTOWER, LightCycler® 96 or 480 I).

Content

1. Intended Use 3
2. Pathogen Information 3
3. Testing Principle 3
4. Package Content 3
5. Additional Equipment and Reagents 4
6. Transport, Storage and Stability 4
7. Warnings and Safety Precautions 4
8. Important Points before Starting/Sample Material 5
9. Procedure 5
10. Instrument Settings 5
11. Results 6
12. Limitations of the Method 6
13. Analytical Performance 7
13.1 Analytical Sensitivity 7
13.2 Analytical Specificity 7
14. Diagnostic Performance 8
15. Note to the User 9
16. Symbols 9

1. Intended Use

The Norovirus *direct* RT-PCR test is an in vitro real-time reverse transcription polymerase chain reaction (rRT-PCR) test for the qualitative detection and differentiation of norovirus (genogroup I and II) in human stool samples. The results support the diagnosis of gastroenteritis. The test is intended for use in qualified laboratories by personnel trained in molecular diagnostic techniques.

2. Pathogen Information

Noroviruses cause the most cases of non-bacterial gastroenteritis outbreaks.[1] A gastroenteritis caused by norovirus is characterised by severe nausea, vomiting and diarrhea.[2] The viruses are transmitted by fecally contaminated food or water and by person-to-person contact which is often the cause of a rapid spreading in shared facilities.

Noroviruses are small non-enveloped single-stranded RNA (ssRNA) viruses belonging to the family of Caliciviridae. They can be grouped in 7 genogroups with over 30 genotypes. To date, human pathogens have only been described from genogroup I (GI) with 9 genotypes, from genogroup II (GII) with 22 genotypes and from genogroup IV (GIV) with two genotypes.[3]

3. Testing Principle

The Norovirus *direct* RT-PCR test is designed to detect norovirus RNA after extraction of RNA from biological samples on a commercially available qPCR cycler. It consists of a test kit with solutions that contain all necessary chemicals (buffer components, substances for reverse transcription and PCR including an internal process control). After adding extracted RNA to the reaction solution, the RT-PCR analysis is carried out in the qPCR device.

The Norovirus *direct* RT-PCR test contains probes specific for the following targets: (1) Norovirus GI, (2) Norovirus GII, and (3) Internal Control. The probes are each labelled with fluorescent reporter dyes and a second dye that serves as a quencher and suppresses the fluorescence signals of the intact probes. The analysis is performed by determining Ct (cycle threshold) values. The Ct value describes the cycle in which the signal rises above a certain threshold for the first time. The more target copies (here: virus RNA) are present in the sample, the lower the Ct value. The Internal Control (IC) allows the detection of RT-PCR inhibition and may also act as control for nucleic acid isolation from the biological specimen. The buffer contains additives for enhancement of PCR efficiency and inhibition of RNases.

4. Package Content

The reagents of each vial set are sufficient for 96 reactions. Each set contains:

Material	Lid Colour	Comment
Solution A	blue	Reaction buffer, enzymes, primers and probes
Solution B	yellow	Internal Control (artificial RNA target)
Negative Control	colourless	RNA/DNA-free molecular grade water
Positive Control	green	Norovirus GI- and GII-specific RNA

Additionally, a quick start protocol is provided per packing unit.

[1] Campillay-Véliz, C.P. et al. Human Norovirus Proteins: Implications in the Replicative Cycle, Pathogenesis, and the Host Immune Response. Front. Immunol., 16 June 2020

[2] 16 May 2022: https://www.rki.de/DE/Content/Infekt/EpidBull/Merkblaetter/Ratgeber_Noroviren.html

[3] Chhabra, P. et al. Updated classification of norovirus genogroups and genotypes. Journal of General Virology 2019;100:1393–1406

5. Additional Equipment and Reagents

- qPCR cycler
- disposable protective gloves, powder-free
- PCR reaction tubes/microtiter plate plus lids/adhesive optical film
- Pipettes
- Pipette tips with filter (DNase/RNase-free)
- Table centrifuge
- RNA isolation kit

6. Transport, Storage and Stability

The Norovirus *direct* RT-PCR test is shipped on dry ice. All components must be stored at -25 °C to -18 °C in the dark immediately after receipt. Reagents should be handled at +2 °C to +8 °C and used within 4 hours. Repeated thawing and freezing of the components – more than once – shall be avoided. If necessary, aliquoting of the test components after the first thawing is recommended. The package bears an expiry date, after which no quality guarantee can be given.

7. Warnings and Safety Precautions

The Norovirus *direct* RT-PCR test is intended for in vitro diagnostic use only. The test should only be performed by personnel trained in molecular diagnostic techniques. Cross-contamination can lead to false test results. Add patient samples and controls carefully. Ensure that reaction preparations are not carried over from one well to the next. If the user makes substantial changes to the product or the application instructions, results may not correlate with the intended use.

- Before performing the test, read the entire instructions for use and follow them carefully. Deviations from the given test protocols can lead to incorrect results.
- All patient samples must be treated as potentially infectious material.
- Discard sample and assay waste (that was in contact with patient material) according to your local, regional, or national safety regulations.
- The concentration specifications and incubation times of the manufacturers must be followed.
- Do not use the test beyond the expiration date.
- Do not use the test with opened or damaged packaging.
- Protect reagents from heat, moisture, and light.
- Do not replace or mix the reagents with reagents from other batches or other chemicals.
- Avoid contamination of the test by microorganisms and nucleases (DNases and RNases).
- Any carry-over of samples during handling and processing of the test may result in false positive test results.
- Good laboratory practice should be followed during the test.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner.
- Always wear disposable gloves in each area and change them before entering a different area.
- The test kits are intended for single use and must not be reused.
- If contamination of the qPCR device is suspected, cleaning and maintenance must be carried out according to the qPCR system manual.
- Safety Data Sheets (SDS) are available on request from FRIZ Biochem.

8. Important Points before Starting/Sample Material

Follow the procedures in these instructions for use: reliable results can only be guaranteed if proper procedures are used for sampling, transportation, storage, and handling of the samples. Good laboratory practice is necessary to avoid contamination of the reagents.

The Norovirus *direct* RT-PCR test is intended for the detection of norovirus RNA in human stool samples. Other specimen types may cause false negative or invalid results.

The presence of various interfering substances can lead to false negative or invalid results. The Norovirus *direct* RT-PCR test includes an Internal Control (artificial RNA target) to detect samples containing substances that interfere with RNA isolation, reverse transcription, and PCR amplification.

In every RT-PCR run one positive control and one negative control should be included. The Positive Control FBC113-PC consists of norovirus GI- and GII-specific RNA. A failed Positive or Negative Control will invalidate the RT-PCR run and the results must not be reported.

The starting material for the Norovirus *direct* RT-PCR test is RNA isolated from human stool samples. This assay must not be used on a specimen directly. Appropriate nucleic acid extraction methods must be conducted prior to using this assay. RNA extraction reagents are not part of the Norovirus *direct* RT-PCR test.

9. Procedure

Thaw all reagents directly before starting the test, keep them cool (+2 °C to +8 °C), and use within 4 hours.

	Procedure with RNA extraction
1	Add 100 µl of **Solution B** to the lysis buffer per extraction run in a 96-well plate format.
2	Perform RNA extraction according to your laboratory's standard procedure.
3	Pipette 15 µL of **Solution A** into each PCR reaction tube/well of the microtiter plate.
4	Add 10 µL of **Positive Control** and 10 µL of **Negative Control** per run in respective wells. Add 10 µL of **eluate from RNA extraction** per remaining reaction tube/well of the microtiter plate.
5	Close the microtiter plate with an adhesive optical film or the reaction tubes with the lids provided.
6	Briefly centrifuge the microtiter plate or reaction tubes.
7	Place the filled plate/reaction tubes in the qPCR cycler.

10. Instrument Settings

The Norovirus *direct* RT-PCR test was validated with the Roche LightCycler® 480 II and with the BioRad qPCR cycler CFX Opus 96™. In general, FRIZ *direct* PCR tests are compatible with any qPCR cycler (e.g., MIC Magnetic Induction Cycler, ABI QuantStudio 5, AJ qTOWER, Roche LightCycler® 96, Roche LightCycler® 480 I) but these and other cyclers may lead to altered Ct values and results. For basic information on programming your qPCR cycler, please refer to the user manual of the qPCR cycler used. The following thermal profile is to be used.

Steps	Temperature [°C]	Time	Number of cycles
Reverse transcription	55	10 min	1x
Initial denaturation	95	2 min	1x
Denaturation	95	10 sec	45x
Amplification/Elongation	60	30 sec	

Channel settings for a qPCR cycler are given below.

	Norovirus GI	Norovirus GII	Internal Control (IC)
Reporter dye	FAM	Red 610	HEX
Colour	green	orange	yellow-green
Emission	520 nm	610 nm	560 nm
Quencher	Black Hole Quencher		

11. Results

Positive samples show a qPCR typical amplification curve that crosses a certain threshold generating the Ct value.

- The Negative Control must not show amplification curves in all channels except for the IC channel.
- The Positive Control must show amplification curves in all channels. The Ct value of the Positive Control must be < 36. A Positive Control with a higher Ct value indicates procedural problems.
- The IC for negative samples must show a positive amplification curve with a Ct value comparable to the IC Ct value in the Negative Control.
- The IC for positive samples should show a positive amplification curve with a Ct value comparable to the IC Ct value in the Negative Control.
- A significantly higher IC Ct value in samples compared to the IC Ct value in the Negative Control indicates procedural problems (e.g., purification problems or RT-PCR inhibition).
- A high concentration of detectable viral RNA in the sample can lead to reduced or absent IC signals. In such cases the result for the IC can be neglected.

Norovirus GI (FAM)	Norovirus GII (Red 610)	Internal Control (HEX)	Result	Interpretation
Positive	Negative	Positive/ Negative	**Positive**	Norovirus GI detected
Negative	**Positive**	Positive/ Negative	**Positive**	Norovirus GII detected
Positive	**Positive**	Positive/ Negative	**Positive**	Norovirus GI and GII detected
Negative	Negative	**Positive**	**Negative**	Target genes not detected
Negative	Negative	Negative	**Invalid!**	The test result cannot be evaluated.

12. Limitations of the Method

Test results should always be seen in the context of the clinical findings. Therapeutic consequences of the diagnostic results must be drawn in relation to the clinical findings.

The results support the differential diagnosis of infections with norovirus. The viral RNA is generally detectable in human stool samples during the acute phase of the infection. Positive results indicate the presence of the respective RNA, but do not exclude a co-infection with other pathogens.

A negative result does not exclude the presence of noroviruses, as results depend on correct sampling, the absence of inhibitors and sufficient RNA to be detected.

Invalid results may be obtained if the sample contains inhibitors that prevent extraction, transcription, amplification, or detection of the target nucleic acids.

The detection of analyte target does not mean that corresponding viruses are infectious, nor that they are the causative agents of clinical symptoms.

There is a risk of false positive results due to cross-contamination by target organisms, their nucleic acids or their amplified product.

Mutations or polymorphisms in primer and probe binding regions can interfere with the detection of new variants that may result in false negative results.

Norovirus genogroup IV, which very rarely infect humans, will also be detected by the Norovirus *direct* RT-PCR test.

13. Analytical Performance

13.1 Analytical Sensitivity

The limit of detection (LoD) was determined with serial dilutions of quantified target-specific RNA. The analytes were tested in 24 replicates per concentration on a Bio-Rad CFX Opus 96. The 95% detection limit (95 CI) was determined using Logistic Regression (Logit) with the GraphPad Prism 9.3.1 software.

Analytical sensitivity (LoD $_{95\ CI}$):	**Norovirus GI: LoD$_{95\ CI}$ < 30 copies/reaction** **Norovirus GII: LoD$_{95\ CI}$ < 30 copies/reaction**

13.2 Analytical Specificity

In silico analyses of primers and probes showed no cross-reactivities to sequences of other gastrointestinal pathogens.

In addition to *in silico* analyses, the specificity of the test was determined *in vitro* by examining inactivated gastrointestinal viral and bacterial isolates in a stool diluent that mimics the composition of a true clinical specimen. The measurement was performed in triplicates. The following table gives an overview of the microorganisms tested for cross-reactivity:

Organism	Strain	Result
Salmonella enterica typhimurium	Z005	Negative
Escherichia coli	EDL933; O157	Negative
Escherichia coli	92.0147; EAEC	Negative
Escherichia coli	ETEC; ST+, LT+	Negative
Escherichia coli	7.1493; O84:H28; EPEC	Negative
Shigella sonnei	Z004	Negative
Entamoeba histolytica	DS4-868	Negative
Vibrio cholerae	Z133; non-toxigenic	Negative
Cryptosporidium parvum	Iowa	Negative
Giardia lamblia	H3	Negative
Clostridium difficile	NAP1	Negative
Campylobacter jejuni	Clinical isolate	Negative
Campylobacter coli	Clinical isolate	Negative
Yersinia enterocolitica	Clinical isolate	Negative
Pleisomonas shigelloides	Z130	Negative
Cyclospora cayetanensis	recombinant	Negative
Astrovirus	recombinant	Negative
Sapovirus	recombinant	Negative
Rotavirus	Wa	Negative
Adenovirus Type 41	TAK	Negative

None of the pathogens tested gave a positive signal in the Norovirus *direct* RT-PCR test (all Internal Controls valid).

14. Diagnostic Performance

The evaluation of the clinical performance was carried out with serial dilutions of different recombinant norovirus genotypes spiked in a stool diluent that mimics the composition of a true clinical specimen. RNA was isolated using the QIAmp Viral RNA mini kit (Qiagen). Negative samples were analysed in parallel with a CE-certified test kit from another manufacturer.

	Norovirus positive samples	Norovirus negative samples
Norovirus *direct* RT-PCR positive	100	0
Norovirus *direct* RT-PCR negative	0	50
Sensitivity/Specificity	**Sensitivity 100%**	**Specificity 100%**

The diagnostic sensitivity of the Norovirus *direct* RT-PCR test is strongly dependent on the RNA extraction method used to isolate RNA from biological specimens. It is the responsibility of the user to qualify the extraction methods used for RNA isolation from biological samples.

15. Note to the User

All serious incidents relating to the device must be notified to the manufacturer and the competent authority of the Member State where the user and/or patient is established.

This product complies with the requirements of the European Directive 98/79/EC

for in vitro diagnostics.

16. Symbols

IVD	For use in in vitro diagnostics	REF	Item number
	Manufacturer		Content sufficient for XY provisions
	Temperature limitation		Biological risk
	Can be used until	LOT	Batch designation
	Follow instructions for use	CONT	Content

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