altona DIAGNOSTICS

Instructions for use

FlexStar®
Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5

05/2022 EN

Gastrointestinal

FlexStar®

FlexStar®

Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5

For use with

CFX96™ Deep Well Dx System (Bio-Rad)

CFX96™ Dx System (Bio-Rad)

QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)

Rotor-Gene® Q5/6 plex Platform (QIAGEN)

CE

IVD

REF FS0071543

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05 2022

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MAN-FS0071540-EN-S01

Content

1. About these instructions for use

Throughout this manual, the terms CAUTION and NOTE have the following meanings:

CAUTION

Highlights operating instructions or procedures which, if not followed correctly, may result in personal injury or impact product performance. Contact altona Diagnostics technical support for assistance.

NOTE

Information is given to the user that is useful but not essential to the task at hand.

Read the instructions for use carefully before using the product.

2. Intended use

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is an *in vitro* diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of norovirus genogroup I (GI), norovirus genogroup II (GII) and rotavirus specific RNA in human stool specimens.

It is intended to be used as an aid for diagnosis of norovirus and rotavirus infection.

The results generated with the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 have to be interpreted in conjunction with other clinical and laboratory findings.

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is intended for use by professional users trained in molecular biological techniques and *in vitro* diagnostic procedures.

3. Product content

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 contains the following components:

Table 1: Kit components

Lid color	Component	Number of tubes	Nominal volume [µl/tube]
Blue	Detection Mix[1)]	8	60
Red	PC[2)]	2	250
White	NTC[3)]	2	250

[1)] Contains biological material of animal origin

[2)] Positive Control (norovirus GI, norovirus GII and rotavirus specific RNA)

[3)] No Template Control (negative control)

CAUTION

Before first use check the product and its components for completeness with respect to number, type and filling. Do not use a defective or incomplete product, product performance could be compromised.

4. Storage

- The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is shipped on dry ice. The product components should arrive frozen. If one or more components are not frozen upon receipt, or if tubes have been compromised during shipment, contact altona Diagnostics technical support for assistance (see chapter 15. Technical assistance).
- All components should be stored at -25 °C to -15 °C upon arrival.
- Repeated thawing and freezing of the Detection Mix component should be avoided, as this might affect the performance of the product.
- Repeated thawing and freezing of the Positive Control (PC) and No Template Control (NTC) (more than 4 times) should be avoided, as this might affect the performance of the product.
- Storage at room temperature (max. +30 °C) should not exceed a period of 2 hours.
- Protect the Detection Mix component from light.

CAUTION

Improper storage conditions could compromise product performance.

CAUTION

Do not exceed thaw-freeze-sequence and handling durations specified in these instructions for use, as this could compromise product performance.

CAUTION

Do not use products beyond the expiration date. The use of expired products could compromise product performance.

5. Background information

Norovirus

The genus *Norovirus* (Norovirus) belongs to the family of *Caliciviridae* and is formerly known as *Norwalk-like virus*. Noroviruses are single stranded RNA viruses, discovered in 1972 by electron-microscopy. They are characterized by their high degree of genomic variability. Noroviruses have been classified into ten genogroups (GI to GX) based on sequence by comparison of the RNA polymerase and capsid region of the genome. Genogroups I, II, and IV are associated with infections in humans. To date, genogroups GI and GII are the most clinically relevant groups and are subdivided into at least 9 and 27 genotypes, respectively.

Noroviruses are responsible for the majority of non-bacterial acute gastroenteritis in humans in industrialized countries. The symptoms of vomiting and diarrhea occur after a short incubation time of 8 to 72 hours. Noroviruses are highly infectious.

Infections with norovirus can either be caused by contaminated food and/or drinking water or person-to-person virus transmission. Norovirus can cause large outbreak situations in settings of close human contact such as hospitals, nursing homes, cruise ships, etc. [1,2,3].

Rotavirus

Rotaviruses belong to the family *Reoviridae* and are divided into the species *A-G*. Species *A-C* are infectious to humans whereby the species *A* (Taxonomie: *Rotavirus A*) is the group which cause >90 % of infections in humans. The genome is segmented dsRNA with 11 segments. Species are classified by comparison of the sequence of the segment VP6. The rotavirus genome is very heterogeneous in all segments. Rotavirus A is subdivided in several genotypes.

Rotaviruses are found as the causative agent in a high number (>500,000; WHO) of fatal diarrhoea cases in children under the age of 5 worldwide, especially in developing countries. But also children above the age of 5 and adults can be affected by this pathogen, with a severe course and even fatal cases. Mostly, the fatal cases are due to a lack of medical service and bad hygienic conditions in developing countries. Rotavirus outbreaks occur most often in winter (CDC), with a peak in March (“winter diarrhoea”).

The symptoms are diarrhoea, vomiting, fever and abdominal cramps. Incubation time is 1-3 days and the course lasts about 4-7 days. Rotavirus is transmitted by smear infection, contaminated water and food with a low infectious dose (<100 virus particles). The virus has a high tenacity and the viral load can be up to 10^{10} virus particles/gr stool.

Medical treatment is not available so far and sequelae of an infection can only be reduced by preventive treatment [4,5].

NOTE

Due to the relatively fast molecular evolution of RNA viruses, there is an inherent risk for any RT-PCR based test system that accumulation of mutations over time may lead to false negative results.

6. Product description

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is an *in vitro* diagnostic test. Used in combination with the FlexStar® (RT-)PCR Amplification Mix 1.5 it allows the qualitative detection and differentiation of norovirus genogroup I (GI), norovirus genogroup II (GII) and rotavirus specific RNA in human stool specimens.

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is based on real-time RT-PCR technology, utilizing reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), polymerase chain reaction (PCR) for the amplification of norovirus GI, norovirus GII and rotavirus specific target sequences and fluorescently labeled target specific probes for the detection of the amplified cDNA.

In addition to the norovirus GI, norovirus GII and rotavirus RNA specific amplification and detection systems, the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 includes oligonucleotides for the amplification and detection of the internal control (IC, AltoStar® Internal Control 1.5). The IC is automatically added at the beginning of the nucleic acid purification procedure on the AltoStar® Automation System AM16 (in the following summarized as AltoStar® AM16). For details refer to the instructions for use of the AltoStar® Internal Control 1.5.

Probes specific for norovirus GI RNA are labeled with the fluorophore Cy5, probes specific for norovirus GII RNA are labeled with the fluorophore ROX™ and probes specific for rotavirus RNA are labeled with the fluorophore FAM™, respectively. The probe specific for the IC is labeled with the fluorophore JOE™.

Using probes linked to distinguishable dyes enables the parallel detection of norovirus GI, norovirus GII, rotavirus and the IC in the corresponding detection channels of the real-time PCR instrument.

6.1 Components

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 contains enough reagents for 96 reactions. The product consists of the following components:

- Detection Mix[1)]
- PC[2)]
- NTC[3)]

[1)] Contains biological material of animal origin

[2)] Positive Control (norovirus GI, norovirus GII and rotavirus specific RNA)

[3)] No Template Control (negative control)

Except for the DNA polymerase and the reverse transcriptase, which are included in the FlexStar® (RT-)PCR Amplification Mix 1.5, the Detection Mix component contains all reagents (PCR buffer, magnesium salt, primers and probes) to allow detection and differentiation of norovirus GI and norovirus GII and detection of rotavirus specific RNA, as well as of IC specific RNA.

The PC contains norovirus GI and norovirus GII as well as rotavirus specific RNA. It is used to verify the functionality of the norovirus GI, norovirus GII and rotavirus RNA specific amplification and detection systems.

The NTC contains neither norovirus GI, norovirus GII, nor rotavirus specific RNA but does contain the IC template. The NTC is used as negative control for the norovirus GI, the norovirus GII and the rotavirus RNA specific real-time PCR and indicates possible contamination of the Detection Mix component.

6.2 Real-time PCR instruments

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was developed and validated to be used with the following real-time PCR instruments:

- CFX96™ Deep Well Dx System (Bio-Rad)
- CFX96™ Dx System (Bio-Rad)
- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- Rotor-Gene® Q5/6 plex Platform (QIAGEN)

NOTE

Ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.

6.3 Sample types

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 has been validated for use with the following sample type:

- Human stool

CAUTION

Do not use other sample types! The use of other sample types could compromise product performance.

7. Material required but not provided

The following additional instruments and consumables are required for use of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 but not provided with this product:

- Appropriate real-time PCR instrument (see chapter 6.2 Real-time PCR instruments)
- Appropriate nucleic acid extraction system or kit (see chapter 9.2 Sample preparation)
- Vortex mixer
- Centrifuge (e.g. desktop centrifuge) for centrifugation of kit reagents
- Centrifuge for centrifugation of PCR plates
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material
- Pipettes (adjustable)
- Pipette tips with filters (disposable)
- Powder-free gloves (disposable)

Reagents required but not included in the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5:

- FlexStar® (RT-)PCR Amplification Mix 1.5 (Order No. FS0011515)
- AltoStar® Internal Control 1.5 (Order No. IC15-46)

8. Warnings, precautions and limitations

- Before first use check the product and its components for completeness with respect to number, type and filling. Do not use a defective or incomplete product, product performance could be compromised.
- Improper storage conditions could compromise product performance.
- Do not exceed thaw-freeze-sequence and handling durations specified in these instructions for use, as this could compromise product performance.
- Do not use products beyond the expiration date. The use of expired products could compromise product performance.
- Do not use other sample types! The use of other sample types could compromise product performance.
- Improper handling of product components and samples may cause contamination and could compromise product performance:
  - Do not interchange vial or bottle caps.
  - Store positive and/or potentially positive material separated from the kit components.
  - Use separated working areas for sample preparation/reaction setup and amplification/detection activities.
  - Always dispose gloves after handling positive and/or potentially positive material.
  - Do not open the PCR plates and/or tubes post amplification.
- Do not mix components from different FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 lots, as this could compromise product performance.
- Always treat samples as infectious and (bio-)hazardous material in accordance with safety and laboratory procedures. For sample material spills promptly use an appropriate disinfectant. Handle contaminated materials as biohazardous.
- The presence of PCR inhibitors could cause false negative or invalid results.
- Disposal of hazardous and biological waste shall comply with local and national regulations to avoid environmental contamination.
- Storage of eluates under wrong conditions may lead to degradation of the norovirus GI, norovirus GII and/or rotavirus target sequences and could compromise product performance.

- A lack of centrifugation of the product components after thawing may cause contamination with reagent residues in the lids and could compromise product performance.
- Do not exceed the PCR mix storage time, as this could compromise product performance.
- As with any diagnostic test, results shall be interpreted in consideration of all clinical and laboratory findings.
- In case the sample contains other pathogens than norovirus GI, norovirus GII and/or rotavirus, competition with the target amplification or cross-reactivities may occur, causing incorrect IVD examination results.
- Potential mutations within the target regions of the norovirus GI, norovirus GII and/or rotavirus genome covered by primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.

9. Procedure

CAUTION

Improper handling of product components and samples may cause contamination and could compromise product performance:

- Do not interchange vial or bottle caps.
- Store positive and/or potentially positive material separated from the kit components.
- Use separated working areas for sample preparation/reaction setup and amplification/detection activities.
- Always dispose gloves after handling positive and/or potentially positive material.
- Do not open the PCR plates and/or tubes post amplification.

CAUTION

Do not mix components from different FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 lots, as this could compromise product performance.

9.1 Sample collection, handling and storage

Before use stool suspension samples should not be stored for more than 48 hours at room temperature (+20 °C to +25 °C), 72 hours at +2 °C to +8 °C or 7 days at -25 °C to -15 °C.

CAUTION

Always treat samples as infectious and (bio-)hazardous material in accordance with safety and laboratory procedures. For sample material spills promptly use an appropriate disinfectant. Handle contaminated materials as biohazardous.

NOTE

Frozen storage of samples does not compromise kit performance. When working with frozen samples, make sure samples are completely thawed and properly mixed before use.

9.2 Sample preparation

Extracted RNA is the starting material for the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5. The quality of the extracted RNA has a profound impact on the performance of the product.

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was validated with human stool specimens using the AltoStar® AM16 in combination with the AltoStar® Purification Kit 1.5.

After completion of nucleic acid extraction using the AltoStar® AM16, the eluates in the unsealed eluate plate are stable at room temperature (max. +30 °C) for a total of 4 hours.

The eluates in a sealed eluate plate can be stored at +2 °C to +8 °C for up to 24 hours before the start of a reaction setup. For detailed information regarding the sealing of eluate plates, refer to the instructions for use of the AltoStar® Purification Kit 1.5.

Alternative nucleic acid extraction systems and kits might also be appropriate.

However, the suitability of the nucleic acid extraction procedure for use with the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 has to be validated by the user.

CAUTION

Do not use other sample types! The use of other sample types could compromise product performance.

CAUTION

Always treat samples as infectious and (bio-)hazardous material in accordance with safety and laboratory procedures. For sample material spills promptly use an appropriate disinfectant. Handle contaminated materials as biohazardous.

CAUTION

The presence of PCR inhibitors could cause false negative or invalid results.

CAUTION

Disposal of hazardous and biological waste shall comply with local and national regulations to avoid environmental contamination.

CAUTION

Storage of eluates under wrong conditions may lead to degradation of the norovirus GI, norovirus GII and/or rotavirus target sequences and could compromise product performance.

For additional information and technical support regarding pre-treatment and sample preparation, contact altona Diagnostics technical support (see chapter 15. Technical assistance).

9.3 Master mix setup

All reagents and samples should be thawed completely, mixed (by pipetting or gentle vortexing) and centrifuged briefly before use.

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is configured for use with the FlexStar® (RT-)PCR Amplification Mix 1.5 and the AltoStar® Internal Control 1.5, which allows to control the sample preparation procedure (nucleic acid extraction) and the subsequent RT-PCR.

- ► The IC is automatically added at the beginning of the nucleic acid purification procedure on the AltoStar® AM16.
- ► When using other nucleic acid extraction methods, the IC has to be added during the lysis step either manually or automatically by the respective instrument.
- ► No matter which method/system is used for nucleic acid extraction, never add the IC directly to the sample. The IC should always be added to the sample/lysis buffer mixture. The volume of the IC which has to be added, always and only depends on the elution volume. It represents 50 % of the elution volume. For instance, if the nucleic acid is going to be eluted in 60 µl of elution buffer or water, 30 µl of IC per sample must be added into the sample/lysis buffer mixture.
- ► Set up the master mix according to the following pipetting scheme:

Table 2: Pipetting scheme (master mix setup)

Number of reactions (rxns)	1	12
Detection Mix	5 µl	60 µl
Amplification Mix	15 µl	180 µl
Volume master mix	**20 µl**	**240 µl**

CAUTION

A lack of centrifugation of the product components after thawing may cause contamination with reagent residues in the lids and could compromise product performance.

9.4 Reaction setup

- ► Pipette 20 µl of the master mix into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.
- ► Add 10 µl of the sample (eluate from the nucleic acid extraction) or 10 µl of the controls (PC or NTC).

Table 3: Pipetting scheme (reaction setup)

Reaction setup	
Master mix	20 µl
Sample or control	10 µl
Total volume	**30 µl**

- ► Make sure that at least 1 PC and 1 NTC is used per run.
- ► Thoroughly mix the samples and controls with the master mix by pipetting up and down.
- ► Close the 96-well reaction plate with appropriate lids or optical adhesive film and the reaction tubes with appropriate lids.
- ► Centrifuge the 96-well reaction plate in a centrifuge with a microtiter plate rotor and the reaction tubes in an appropriate centrifuge for 30 seconds at approximately 1,000 x g (~ 3,000 rpm).
- ► The NTC does already contain the IC template in the correct concentration.

After completion of the PCR mix setup the RT-PCR mix in a sealed PCR plate is stable at room temperature (max. +30 °C) for max. 30 minutes.

CAUTION

Do not exceed the PCR mix storage time, as this could compromise product performance.

10. Programming the real-time PCR instrument

For basic information regarding the setup and programming of the different real-time PCR instruments, refer to the user manual of the respective instrument.

For detailed programming instructions regarding the use of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 on specific real-time PCR instruments, contact altona Diagnostics technical support (see chapter 15. Technical assistance).

10.1 Settings

► Define the following settings:

Table 4: Run settings

Settings	
Reaction volume	30 µl
Ramp rate	Default
Passive reference	None

10.2 Fluorescence detectors (dyes)

► Define the fluorescence detectors (dyes):

Table 5: Fluorescence detectors

Target	Detector name	Reporter	Quencher
Norovirus GI specific RNA	Norovirus GI	Cy5	(None)
Norovirus GII specific RNA	Norovirus GII	ROX™	(None)
Rotavirus specific RNA	Rotavirus	FAM™	(None)
Internal Control	Internal Control	JOE™	(None)

10.3 Temperature profile and dye acquisition

► Define the temperature profile and dye acquisition:

Table 6: Temperature profile and dye acquisition

	Stage	Cycle repeats	Acquisition	Temperature [°C]	Time [min:s]
Reverse transcription	Hold	1	-	52	05:00
Denaturation	Hold	1	-	95	00:05
Amplification	Cycling	45	-	95	00:05
			Yes	58	00:25

11. Data analysis

For basic information regarding data analysis on specific real-time PCR instruments, refer to the user manual of the respective instrument.

For detailed instructions regarding the analysis of the data generated with the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 on different real-time PCR instruments, contact altona Diagnostics technical support (see chapter 15. Technical assistance).

11.1 Validity of diagnostic test runs

11.1.1 Valid diagnostic test run

A diagnostic test run is **valid**, if the following control conditions are met:

Table 7: Control conditions for a valid test run

Control ID	Detection channel			
	Cy5	ROX™	FAM™	JOE™
Positive Control (norovirus GI, norovirus GII and rotavirus specific RNA)	+	+	+	Not applicable
Negative control	-	-	-	+

11.1.2 Invalid diagnostic test run

A diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an **invalid** diagnostic test run, repeat testing by using the remaining purified nucleic acids or start from the original samples again.

11.2 Interpretation of results

CAUTION

As with any diagnostic test, results shall be interpreted in consideration of all clinical and laboratory findings.

11.2.1 Qualitative analysis

Table 8: Result interpretation

Detection channel				Result interpretation
Cy5 (norovirus GI)	ROX™ (norovirus GII)	FAM™ (rotavirus)	JOE™ (IC)	
+	+	-	+/-*	Norovirus GI and norovirus GII specific RNA detected.
+	-	-	+/-*	Only norovirus GI specific RNA detected.
-	+	-	+/-*	Only norovirus GII specific RNA detected.
-	-	+	+/-*	Only rotavirus specific RNA detected.
+	-	+	+/-*	Norovirus GI and rotavirus specific RNA detected.
-	+	+	+/-*	Norovirus GII and rotavirus specific RNA detected.
+	+	+	+/-*	Norovirus GI, norovirus GII and rotavirus specific RNA detected.
-	-	-	+	Neither norovirus GI, nor norovirus GII, nor rotavirus specific RNA detected. The sample does not contain detectable amounts of norovirus GI, norovirus GII or rotavirus specific RNA.
-	-	-	-	RT-PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

* Detection of the IC in the JOE™ detection channel is not required for positive results in the Cy5 and/or the ROX™ and/or the FAM™ detection channel. A high norovirus GI and/or norovirus GII and/or rotavirus RNA load in the sample can lead to reduced or absent IC signals.

12. Performance evaluation

The performance of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was evaluated using norovirus GI, norovirus GII and rotavirus commercially available virus material.

12.1 Stool

12.1.1 Analytical sensitivity

For the determination of the limit of detection (LoD) for norovirus GI and norovirus GII a dilution series of commercially available virus material in artificial stool was generated. For the determination of the LoD for rotavirus a dilution series of commercially available virus material in stool suspension was generated.

Each dilution was tested in 8 replicates in 3 different runs (total n = 24 per dilution) using combinations of:

- 3 FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 lots
- 3 FlexStar® (RT-)PCR Amplification Mix 1.5 lots
- 3 AltoStar® Purification Kit 1.5 lots
- 3 AltoStar® Internal Control 1.5 lots
- 3 AltoStar® AM16 instruments
- 3 CFX96™ DW Dx instruments

For each virus the data from all runs were combined and a probit analysis was performed to determine the 95 % LoD value.

Table 9: PCR results used for the calculation of the analytical sensitivity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for norovirus GI in artificial stool suspension

Concentration [copies/ml]	N [total]	N [positive]	Hit rate [%]
1.00E+05	24	24	100
3.16E+04	24	24	100
1.00E+04	24	24	100
3.16E+03	24	21	88
1.00E+03	24	11	46
3.16E+02	24	0	0
1.00E+02	24	2	8
3.16E+01	24	0	0
1.00E+01	24	0	0

The LoD of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for the detection of norovirus GI in artificial stool suspension is 5,939 copies/ml (95 % confidence interval: 3,673–12,942 copies/ml).

Table 10: PCR results used for the calculation of the analytical sensitivity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for norovirus GII in artificial stool suspension

Concentration [copies/ml]	N [total]	N [positive]	Hit rate [%]
1.00E+05	24	24	100
3.16E+04	24	24	100
1.00E+04	24	24	100
3.16E+03	24	24	100
1.00E+03	24	17	71
3.16E+02	24	6	25
1.00E+02	24	4	17
3.16E+01	24	4	17
1.00E+01	23*	1	4

* One sample was not processed.

The LoD of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for the detection of norovirus GII in artificial stool suspension is 5,228 copies/ml (95 % confidence interval: 2,790–13,337 copies/ml).

Table 11: PCR results used for the calculation of the analytical sensitivity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for rotavirus in stool suspension

Concentration [copies/ml]	N [total]	N [positive]	Hit rate [%]
1.00E+05	24	24	100
3.16E+04	24	24	100
1.00E+04	24	23	96
3.16E+03	24	14	58
1.00E+03	24	3	13
3.16E+02	24	1	4
1.00E+02	24	0	0
3.16E+01	24	0	0

The LoD of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for the detection of rotavirus in stool suspension is 11,133 copies/ml (95 % confidence interval: 7,126–23,522 copies/ml).

12.1.2 Analytical specificity

The analytical specificity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is ensured by the thorough selection of the oligonucleotides (primers and probes). The oligonucleotides were checked by sequence comparison analysis against publicly available sequences to ensure that all relevant norovirus and rotavirus genotypes will be detected.

For the verification of the analytical specificity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 the following experiments were performed (see chapters 12.1.2.1 Negative samples to 12.1.2.3 Cross-reactivity).

12.1.2.1 Negative samples

32 norovirus GI, norovirus GII and rotavirus negative stool samples from individual donors were tested with the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5. All (32 out of 32) samples were tested negative for norovirus GI, norovirus GII and rotavirus specific RNA and positive for the IC. The analytical specificity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 for stool samples is ≥ 95 %.

12.1.2.2 Interfering substances

To evaluate the influence of potentially interfering endogenous and exogenous substances on the performance of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5, selected substances were spiked in artificial stool suspension containing norovirus GI, norovirus GII and rotavirus in a concentration of the 3 x LoD (17,817 copies/ml, 15,684 copies/ml and 33,399 copies/ml, respectively) and in artificial stool suspension not containing norovirus GI, norovirus GII and rotavirus.

Results obtained for samples containing potentially interfering substances were compared to results generated for artificial stool suspension containing no spiked interferent. Each sample was processed in 3 replicates.

No interference was observed for samples containing elevated levels of:

- Endogenous substances
  - Cholesterol
  - Human genomic DNA
  - Human whole blood
  - Mucin
  - Triglycerides
- Exogenous substances
  - Benzalkonium chloride
  - Ethanol

- Hydrocortison
- Mesalazine
- Naproxen sodium
- Nystatin

CAUTION

The presence of PCR inhibitors could cause false negative or invalid results.

12.1.2.3 Cross-reactivity

The analytical specificity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 with respect to cross-reactivity with other pathogens than norovirus GI, norovirus GII and rotavirus was evaluatted by testing:

- Pathogens related to norovirus GI, norovirus GII and rotavirus
- Pathogens causing similar symptoms as an infection with norovirus GI, norovirus GII or rotavirus
- Pathogens likely to be present in patients suffering from a norovirus GI, norovirus GII or rotavirus infection

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 did not cross-react with any of the following pathogens:

- Astrovirus
- *Campylobacter coli*
- *Campylobacter jejuni*
- *Clostridium difficile*
- Enterohemorrhagic *Escherichia coli* (EHEC)
- *Escherichia coli*
- *Salmonella enterica*
- Sapovirus
- *Vibrio cholerae*

Additionally, norovirus GI, norovirus GII and rotavirus were tested. The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 did not generate false positive signals either in the norovirus GI specific detection channel (Cy5) when testing norovirus GII and rotavirus, the norovirus GII specific detection channel (ROX™) when testing norovirus GI and rotavirus or in the rotavirus specific detection channel (FAM™) when testing norovirus GI and norovirus GII.

CAUTION

In case the sample contains other pathogens than norovirus GI, norovirus GII and/or rotavirus, competition with the target amplification or cross-reactivities may occur, causing incorrect IVD examination results.

12.1.3 Precision

Precision of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was evaluated using a panel consisting of:

- 1 norovirus GI high positive [50 x LoD (2.97E+05 copies/ml)] artificial stool suspension sample
- 1 norovirus GII high positive [50 x LoD (2.61E+05 copies/ml)] artificial stool suspension sample
- 1 rotavirus high positive [50 x LoD (5.57E+05 copies/ml)] artificial stool suspension sample
- 1 norovirus GI low positive [3 x LoD (1.78E+04 copies/ml)] artificial stool suspension sample
- 1 norovirus GII low positive [3 x LoD (1.57E+04 copies/ml)] artificial stool suspension sample
- 1 rotavirus low positive [3 x LoD (3.34E+04 copies/ml)] artificial stool suspension sample
- 1 norovirus GI, norovirus GII and rotavirus negative artificial stool suspension sample

Each panel member was tested in 6 replicates per run.

5 runs were performed on 5 different days using combinations of:

- 3 FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 lots
- 3 FlexStar® (RT-)PCR Amplification Mix 1.5 lots
- 3 AltoStar® Purification Kit 1.5 lots
- 3 AltoStar® Internal Control 1.5 lots
- 3 AltoStar® AM16 instruments
- 3 CFX96™ DW Dx instruments

Repeatability (intra-run variability), inter-lot variability and reproducibility (total variability) were determined based on:

- Threshold cycle (C_q*) values for the norovirus GI, norovirus GII and rotavirus high positive samples (see tables 12–14)
- Threshold cycle (C_q*) values for the IC in the norovirus GI, norovirus GII and rotavirus negative samples (see table 15)

* Please note that the chosen term C_q is equivalent to the designation of C_t, which might be used by other cyclers than the CFX96™ Deep Well Dx System (Bio-Rad).

Table 12: Precision data (CV % based on C_q values) for high positive norovirus GI artificial stool suspension samples

	Norovirus GI high positive sample (CV % based on C_q values)
Intra-run variability	0.26–0.78
Inter-lot variability	0.97
Total variability	0.81

All samples tested at 3 x LoD (low positive samples) were detected positive for norovirus GI.

Table 13: Precision data (CV % based on C_q values) for high positive norovirus GII artificial stool suspension samples

	Norovirus GII high positive sample (CV % based on C_q values)
Intra-run variability	0.28–0.80
Inter-lot variability	2.26
Total variability	1.85

All samples tested at 3 x LoD (low positive samples) were detected positive for norovirus GII.

Table 14: Precision data (CV % based on C_q values) for high positive rotavirus artificial stool suspension samples

	Rotavirus high positive sample (CV % based on C_q values)
Intra-run variability	0.43–0.64
Inter-lot variability	0.66
Total variability	1.61

All samples tested at 3 x LoD (low positive samples) were detected positive for rotavirus.

Table 15: Precision data (CV % based on C_q values) for the IC in norovirus GI, norovirus GII and rotavirus negative artificial stool suspension samples

	IC
Intra-run variability	0.81–1.28
Inter-lot variability	1.48
Total variability	2.10

12.1.4 Total failure rate

The robustness of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was assessed by testing 33 norovirus GI and norovirus GII and 32 rotavirus negative human stool samples from individual donors spiked with norovirus GI, norovirus GII and rotavirus to a final concentration of the 3 x LoD (1.78E+04 copies/ml, 1.57E+04 copies/ml and 3.34E+04 copies/ml). All (33 out of 33 and 32 out of 32) samples were tested positive in the norovirus GI and norovirus GII specific fluorescence detection channels (Cy5 and ROX™, respectively) and in the rotavirus specific fluorescence detection channel (FAM™).

12.1.5 Carry over

Carry over is mostly a workflow dependent risk and independent of the PCR assay used. For the AltoStar® Workflow the AltoStar® Parvovirus B19 PCR Kit 1.5 was used as exemplary model. Potential cross-contamination through carry over from high positive samples was evaluated by testing alternating parvovirus B19 high positive (1.00E+07 IU/ml) and negative samples (n = 44 each per run; 2 runs) with the AltoStar® Parvovirus B19 PCR Kit 1.5. No carry over was observed, i.e. all parvovirus B19 negative samples were tested negative.

12.1.6 Clinical performance

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was evaluated in a comparative study with the CE-marked Allplex™ GI-Virus Assay (Seegene). Retrospectively, 99 individual human stool samples were tested in parallel:

The Allplex™ GI-Virus Assay (Seegene) was used in combination with the STARMag 96 X 4 (Seegene) and the NIMBUS (Seegene).

The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 was used in combination with the FlexStar® (RT-)PCR Amplification Mix 1.5, the AltoStar® Purification Kit 1.5 and the AltoStar® Internal Control 1.5 on the AltoStar® AM16 and the CFX96™ DW Dx.

For the qualitative analysis all samples with an invalid result for one or both assays were excluded.

Results for the remaining samples (94 for norovirus GI and norovirus GII and 96 for rotavirus) are shown in tables 16 and 17, respectively.

Table 16: Results of the evaluation of the diagnostic sensitivity and specificity for norovirus GI and norovirus GII in stool samples

		Allplex™ GI-Virus Assay (Seegene)	
		POSITIVE	NEGATIVE
FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5	POSITIVE	38	0
	NEGATIVE	0	56

The diagnostic sensitivity and specificity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 compared to the Allplex™ GI-Virus Assay (Seegene) for norovirus GI and norovirus GII were 100 % (confidence intervals 90.26 %–100 % and 93.62 %–100 %, respectively).

Table 17: Results of the evaluation of the diagnostic sensitivity and specificity for rotavirus in stool samples

		Allplex™ GI-Virus Assay (Seegene)	
		POSITIVE	NEGATIVE
FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5	POSITIVE	22	0
	NEGATIVE	0	74

The diagnostic sensitivity and specificity of the FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 compared to the Allplex™ GI-Virus Assay (Seegene) for rotavirus were 100 % (confidence intervals 84.56 %–100 % and 95.14 %–100 %, respectively).

13. Disposal

Dispose of hazardous and biological waste in compliance with local and national regulations. Leftover product components and waste should not be allowed to enter sewage, water courses or the soil.

CAUTION

Always treat samples as infectious and (bio-)hazardous material in accordance with safety and laboratory procedures. For sample material spills promptly use an appropriate disinfectant. Handle contaminated materials as biohazardous.

CAUTION

Disposal of hazardous and biological waste shall comply with local and national regulations to avoid environmental contamination.

14. Quality control

In accordance with the altona Diagnostics GmbH EN ISO 13485-certified Quality Management System, each lot of FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is tested against predetermined specifications to ensure consistent product quality.

15. Technical assistance

For customer support, contact altona Diagnostics technical support:

e-mail: **support@altona-diagnostics.com**

phone: **+49-(0)40-5480676-0**

NOTE

Any serious incident that has occurred in relation to this product shall be reported to altona Diagnostics and the competent authority of your country.

16. Literature

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17. Trademarks and disclaimers

AltoStar®, FlexStar® (altona Diagnostics); QuantStudio™ (Applied Biosystems); CFX96™ (Bio-Rad); JOE™ (Life Technologies); Rotor-Gene® (QIAGEN); Allplex™ (Seegene); FAM™, ROX™ (Thermo Fisher Scientific).

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The FlexStar® Norovirus Type & Rotavirus RT-PCR Detection Mix 1.5 is a CE-marked diagnostic kit according to the European *in vitro* diagnostic directive 98/79/EC.

Product not licensed with Health Canada and not FDA cleared or approved.

Not available in all countries.

18. Explanation of symbols

Symbol	Explanation
IVD	*In vitro* diagnostic medical device
GTIN	Global Trade Item Number
LOT	Batch code
CONT	Content
CAP	Cap color
REF	Catalogue number
NUM	Number
COMP	Component
	Consult instructions for use
	Contains sufficient for "n" tests/reactions (rxns)
	Temperature limit
	Use-by date
	Manufacturer
	Caution
MAT	Material number

Symbol	Explanation
	Version
	Note
	Contains biological material of animal origin

19. Revision history

Table 18: Revision history

Identifier	Date of issue [month/year]	Modifications
MAN-FS0071540-EN-S01	05/2022	Initial release

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COV-FlexStar-DM-Gastro-CE-EN-01_07/2021