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Manual

MutaPLEX® Norovirus

real time RT-PCR kit

*Test for the in vitro detection of norovirus RNA (genogroup I
and genogroup II) in stool samples*

Valid from 2016-12-01



KG190132
KG190196



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1 INTENDED USE

The MutaPLEX® Norovirus 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 MutaPLEX® Norovirus real time RT-PCR-Kit 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 MutaPLEX® Norovirus real time RT-PCR kit contains specific primers and dual-labelled 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 an 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, MutaPLEX® Norovirus real time RT-PCR kit contains a control RNA (K5), which is added during RNA extraction and detected in the same reaction by a differently labelled probe.

The control RNA (K5) allows the detection of RT-PCR inhibition and acts as control for the isolation of the nucleic acid from the clinical specimen.

The fluorescence of the control RNA (K5) is measured in the VIC®/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 32 (KG190132) or 96 (KG190196) reactions, respectively.

Table 1: Components of the MutaPLEX® Norovirus real time RT-PCR kit.

	Label	Lid Colour	Content	
			32	96
K1	Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
K2	Enzyme	blue	1 x 6.4 µl	1 x 19.2 µl
K3	Positive control	red	1 x 50 µl	1 x 100 µl
K4	Negative control	green	1 x 50 µl	1 x 100 µl
K5	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. MutaCLEAN® Universal RNA/DNA, KG1038)
- 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 see chapter 11 for details).

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® Norovirus 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 MutaPLEX® Norovirus 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 instructions 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.
- Regularly decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine MutaPLEX® Norovirus real time RT-PCR-Kit components of different lot numbers.

9 SAMPLE MATERIAL

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

10 SAMPLE PREPARATION

The MutaPLEX® Norovirus 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 MutaCLEAN® Universal RNA/DNA (KG1038) are recommended.

Stool samples

Suspend some stool sample (approx. the size of a pea) in 1.5 ml ultra pure water. Vortex well. Once the solid components have sunken to the bottom of the tube (where necessary centrifuge for 5 min at 3 500 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 3 500 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 (K5) 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 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 (K5)

A control RNA (K5) 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 (K5) or VLP-RNA used as extraction control

MutaPLEX® Norovirus control RNA (K5) or VLP-RNA is added to the RNA extraction.

Add 5 µl control RNA (K5) 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 (K5) must be added to the lysis buffer of the extraction kit.

b) Control RNA (K5) 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 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 (K3) and one negative control (K4) should be included.
- Before each use, all reagents – except the enzyme (K2) – should be thawed completely at room temperature, thoroughly mixed (do NOT vortex the reaction mix (K1) but mix by pipetting up and down repeatedly), and centrifuged very briefly.
- We recommend to keep reagents and samples at 2–8°C (e.g. on ice or a cooling block) at all times.

12.2 Procedure

If the control RNA (K5) 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 (K5) is solely used to detect possible inhibition of the real time RT-PCR, please follow protocol B.

Protocol A

The control RNA (K5) 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 (K5) was added during RNA extraction)

Volume per reaction	Volume master mix
15.8 µl Reaction Mix (K1)	15.8 µl x (N+1)
0.2 µl Enzyme (K2)	0.2 µl x (N+1)

Protocol B

The control RNA (K5) 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 (K5) is added directly to the master mix)

Volume per reaction	Volume master mix
15.8 µl Reaction Mix (K1)	15.8 µl x (N+1)
0.2 µl Enzyme (K2)	0.2 µl x (N+1)
0.2 µl Control RNA (K5)*	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 (K3) and the negative control (K4) 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

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	No of cycles
Reverse Transcription	10 min	45 °C	1
Initial Denaturation	5 min	95 °C	1
Amplification of DNA			45 Aquisition at the end of this step
Denaturation	10 s	95 °C	
Annealing	40 s	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 MutaPLEX® Norovirus real time RT-PCR.

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

13 DATA ANALYSIS

The norovirus-specific amplification is measured in the FAM channel. The amplification of the control RNA (K5) is measured in the VIC®/HEX/JOE/TET channel.

The 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 (K5) in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of cDNA may reduce or completely inhibit amplification of the control RNA (K5).

- **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 (K5) excludes the possibilities of RNA isolation failure (in case the control RNA (K5) is being used as an extraction control) and/or real time RT-PCR inhibition. If the CT value of a sample differs significantly from the CT value of the water control, a partial inhibition occurred, 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 (K5) was added during RNA isolation and not directly to the PCR master mix, the negative control (K4) is negative in both channels.

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

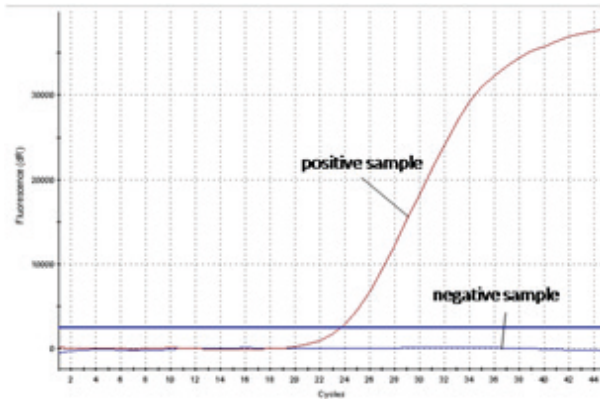


Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

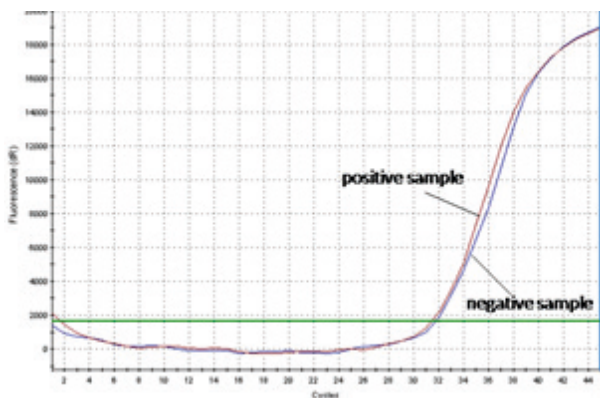


Figure 2: The positive sample as well as the negative sample show a signal in the control RNA-specific VIC®/HEX/JOE/TET channel. The amplification signal of the control RNA (K5) in the negative sample shows that the missing signal in the virus-specific FAM channel is not due to RT-PCR inhibition or failure 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 speci-

mens at high CT – 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 CT of 30.

Internal controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a CT of 33. If the internal control is above CT 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 CT 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 (K3)

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/JOE/TET channel for the amplification of the control RNA (K5).

Incorrect configuration of the real time RT-PCR

Check your work steps and compare with chapter “Procedure”.

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (table 5).

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 chapter “Transport, Storage and Stability”.

Weak or no signal of the control RNA (K5) 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 (chapter 12).

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 (K5) 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 chapter “Transport, Storage and Stability”.

Detection of a fluorescence signal in the FAM channel of the negative control (K4)***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 occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control (K3) 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 MutaPLEX® Norovirus real time RT-PCR kit, 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
MutaPLEX® Norovirus positive	45	0
MutaPLEX® Norovirus negative	0	50
Sensitivity	100 %	
Specificity	100 %	

Table 8: Diagnostic Specificity

Virus/Bacterium	No of strains tested	Result
Norovirus	6 + 39 clinical samples	positive
Enterovirus	11	negative
Adenovirus	1	negative
Rotavirus	1	negative
Astrovirus	1	negative
Sapovirus	1	negative
Listeria monocytogenes	1	negative
Campylobacter	1	negative
Citobacter	1	negative
Salmonella	1	negative
E. coli	1	negative

17.2 Analytical Sensitivity

The analytical sensitivity of MutaPLEX® Norovirus real time RT-PCR kit 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 MutaPLEX® Norovirus real time RT-PCR kit.

Panel Code	Matrix	Strain	Dilution Factor	Result MutaPLEX® Norovirus
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

Panel Code	Matrix	Strain	Dilution Factor	Result MutaPLEX® Norovirus
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	CONTROL -	Negative control (K4)
CT	Cycle Threshold	CONTROL RNA IC	Control RNA (K5)
nn	not known	→REF	To be used with
PCR	Polymerase Chain Reaction	REF	Catalog number
RNA	Ribonucleid Acid	Σ	Contains sufficient for <n> test
RT	Reverse Transcrip- tion	🔥	Upper limit of temperature
VLP	Virus-Like Particles	🏭	Manufacturer
VTM	Virus Transport Medium	🕒	Use by
REACTION MIX	Reaction Mix (K1)	LOT	Lot number
ENZYME	Enzyme (K2)	CONT	Content



Positive control
(K3)



Consult instructions for use



In vitro diagnostic
medical device

19. LITERATURE

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