VIR*Seek* Food Norovirus Genogroup I Real-time RT-PCR

TEST KIT FOR NOROVIRUS GENOGROUP I (NOVGI)
QUALITATIVE REAL-TIME RT-PCR FROM FOOD SAMPLES

Cat. No. 5728200201

For 48 real-time RT-PCR reactions

VIR*Seek*

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1 INTRODUCTION

Human noroviruses are enteric viruses responsible for most gastroenteritis outbreaks worldwide. They are highly infectious and pathogenic throughout all groups of the population. A very low infectious dose of only one to ten viral particles combined with high shedding of viruses is responsible for the rapid spread of the disease in uncontrolled outbreak situations. Human noroviruses transmit via the faecal-oral route and get ingested mainly due to faecal contaminations of food, surfaces and in water as well as through direct human interaction. Due to their structural properties noroviruses are very resistant in the environment and can withstand a broad range of physical and chemical treatments.

Noroviruses of genogroup I (NoVGI) are non-enveloped and single stranded RNA viruses. They are part of the larger *Caliciviridae* family. There are five recognized norovirus genogroups; three of which (GI, GII, and GIV) are known to cause diseases in humans. Most noroviruses that infect humans belong to genogroups I and II; genogroup II being the most prevalent amongst humans.

The detection of noroviruses, and specifically genogroup I and II, in food and on food surfaces is therefore key to the control of the disease. The most widely used diagnostic assay for detecting norovirus is real-time RT -PCR.

The VIRSeek Food Norovirus Genogroup I Real-time RT-PCR kit provides all reagents for the rapid detection of the norovirus genogroup I in a number of food products including soft fruits, leaf, stem and bulb vegetables, bottled water, bivalve molluscan shellfish as well as on food surfaces. A viral concentration prior to RNA extraction is required for these sample types.

The VIRSeek Food Norovirus Genogroup I Real-time RT-PCR kit is validated for use with the Agilent AriaMx™, Bio-Rad CFX96 Touch™ and CFX96 Touch™ Deep Well PCR platforms.

The kit is intended to be used by analytical laboratories for quality control/ quality assurance testing, food and agricultural testing and research purposes only (e.g. virological monitoring of production processes).

The kit is not intended for clinical diagnostics and should therefore be regarded as “For Research Use Only”.

1.1 Test Principle

After concentration of viral particles from food matrices and subsequent extraction of viral RNA the VIR*Seek* Food Norovirus Genogroup I Real-time RT-PCR kit can be used for the detection of the target virus. The first step of a real-time RT-PCR is a reverse transcription (RT) of viral RNA to cDNA, which can then be amplified by real-time PCR. For the extraction of RNA we recommend the VIR*Seek* RNA*Extractor* Food kit (see section 1.3.1).

According to ISO guidelines 15216-1:2017 the processes and horizontal methods for detection of hepatitis A virus and norovirus using real-time RT-PCR in food samples require the usage of a process control virus in order to verify the correct virus concentration process from samples.

Process control viruses need to have similar characteristics to the target virus of interest. Therefore the Eurofins GeneScan Technologies GmbH VIR*Seek* Food Murine Norovirus (MNV) Process Control kit in combination with all food-borne virus detection kits (see section 1.3.1) is recommended.

DNA amplification and detection methods take advantage of the nucleotide sequence conservation found in viral genomes that ensures the potential for high specificity and sensitivity in detection of food-borne pathogenic viruses.

By means of specific primers nucleotide sequences of the norovirus genogroup I are amplified during PCR from isolated and reverse-transcribed total cDNA. Primers do not cross-react with transcribed RNA (cDNA) from other common food-borne virus species including norovirus genogroup II, hepatitis A virus, hepatitis E virus, rotavirus, and DNA derived from other relevant biological material.

1.2 Components of the Kit

For real-time RT-PCR: cat. no. 5728200201

- 1x **OligoMix NoVGI**[*], vial with green cap with primers / probes for IPC / NoVGI and IPC-RNA, 530 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 3 times.
- 1x **BasicMix**[*] **VIR*Seek* Food**, vial with white cap, 265 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 3 times.
- 1x **Positive Control NoVGI**, vial with red cap, 100 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 6 times.
- 1x **Negative Control**, vial with transparent cap, 500 µL, store at -20 °C ± 2 °C.

[*] *Aliquot during first usage if small sample numbers are frequently analysed.*

1.3 Additional Equipment, Consumables and Reagents Required

Equipment:

- 1x **Stepper pipette (1 mL),** (e.g. HandyStep® S (Brand®), Eurofins GeneScan Technologies GmbH, cat. no. 5617703401).
- 1x **Single channel pipette (1 mL, 100 µL**), (e.g. Transferpette® S 100 - 1000 µL (Brand®), Eurofins GeneScan Technologies GmbH, cat. no. 5617703301).
- 1x **Single channel pipette (100 µL, 10 µL),** (e.g. Transferpette® S 10 - 100 µL (Brand®), Eurofins GeneScan Technologies GmbH, cat. no. 5617703201).
- 1x **Single channel pipette (up to 10 µL),** (e.g. Transferpette® S, 0.5 - 10 µL (Brand®), Eurofins GeneScan Technologies GmbH, cat. no. 5617703101).
- 1x **Cooling block** for 1.5 mL tubes.
- 1x **96 well cooling block,** (e.g. Blue cooling block 96 well, Eurofins GeneScan Technologies GmbH, cat. no. 5613900501).
- 1x **Vortex mixer**, (e.g. VWR Collection, cat. no. 444-2790).

- **Centrifuge** for microtiter-plates / or -strips - depending on throughput:
  - Capacity of 2x 8-well strips: (e.g. Carl Roth GmbH, Rotilabo® centrifuge with butterfly rotor, cat. no. T465.1).
  - Capacity of 4x 8-well strips: (e.g. Sigma Aldrich Co LLC, MyFuge™ 12 mini centrifuge, cat. no. Z681733-1EA).
  - Capacity of two times 12x 8-well strips: (e.g. Benchmark Scientific, PlateFuge™ microplate microcentrifuge, cat. no. 5613901701).
- **Real-time PCR Thermocycler**:
  - **Agilent AriaMx™** with HEX™, FAM™ and Cy5™ filter set.
  - **Bio-Rad CFX96 Touch™** (CFX Manager™ Software / CFX Maestro™ Software).
  - **Bio-Rad CFX96 Touch™ Deep Well** (CFX Manager™ Software / CFX Maestro™ Software).

Consumables:

- **RNase-free water** (molecular biology grade).
- **DNA- / Nuclease-free pipette tips** with filters, need to be compatible with pipettes used.
- **RNase-free reaction tubes,** 1.5 mL (e.g. DNA LoBind Tubes, Eppendorf, cat. no. 0030108051).
- **RNase-free pipette tips**, need to be compatible with pipettes used.
- **PCR plates or strips**, compatible with thermocycler used.
- **Optical 8-caps strip or equivalent seals,** compatible with thermocycler used.
- **RNase decontaminating reagent** (e.g. RNase AWAY® Carl Roth GmbH, cat. no. A998).
- **DNA degrading agent** (e.g. Roti® Nucleic Acid-free, Carl Roth GmbH, cat. no. HP69).
- **Gloves,** powder free.

1.3.1 Complete Portfolio of the VIR*Seek* Food Solution

- **VIR*Seek* Food Hepatitis A Virus**, cat. no. 5728200101
  - Real-time RT-PCR kit with 48 reactions for rapid detection of hepatitis A virus (HAV) in food samples.
- **VIR*Seek* Food Norovirus Genogroup I**, cat. no. 5728200201
  - Real-time RT-PCR kit with 48 reactions for rapid detection of norovirus genogroup I (NoVGI) in food samples.
- **VIR*Seek* Food Norovirus Genogroup II**, cat. no. 5728200301
  - Real-time RT-PCR kit with 48 reactions for rapid detection of norovirus genogroup II (NoVGII) in food samples.
- **VIR*Seek* Food Murine Norovirus (MNV) Process Control**, cat. no. 5728200401
  - Murine norovirus spiking material (1 mL) and real-time RT-PCR kit with 48 reactions for rapid detection of murine norovirus (MNV) process control virus in food samples.
- **VIR*Seek* RNA*Extractor* Food**, cat. no. 5524400101
  - Kit for extraction of viral RNA via silica-coated magnetic beads from different food products (incl. soft fruits, leaf, stem and bulb vegetables, bottled water and bivalve molluscan shellfish). 48 x 500 µL or 96 x 250 µL sample input volume. All reagents provided.

2 HOW TO USE THIS PRODUCT

Important Notes:

- Store all reagents as indicated in section 1.2.
- Do not use the reagents beyond the expiration dates printed on the labels.
- Never store kit components in the vicinity of samples or post-PCR products.
- Extraction of viruses from different food matrices should be performed according to the ISO 15216-1:2017 protocol.

2.1 General and Safety Precautions

- All samples should be handled with caution as they are potentially infectious.
- NoVGI should not be handled by pregnant women, children, elderly and immuno-compromised individuals due to the high infection risk and potentially fatal health consequences for this group, in particular for the unborn child in case of pregnant women.
- The VIR*Seek* Food Norovirus Genogroup I Real-time RT-PCR kit contains no hazardous components. For more information, please refer to the VIR*Seek* kit MSDS.
- The VIR*Seek* Food Norovirus Genogroup I Real-time RT-PCR kit contains glycerol and propane-1,2-diol which may cause mild skin irritation.

2.2 Working Guidelines

- Comply with Good Laboratory Practice (refer to EN ISO 7218 standard).
- Refer to EN ISO 22174:2005 for the general requirements for the *in-vitro* amplification of nucleic acid sequences.
- Refer to EN ISO 15216-1:2017 for HAV and norovirus detection in food samples.
- Perform cleaning protocol (outlined in section 2.4).
- Use DNA-, nuclease-free, and sterile lab ware.
- Wear gloves and change frequently.

2.3 RNA Handling – Specific Working Guidelines

It is important to create and maintain an RNase-free environment when working with RNA. RNases are very thermostable enzymes degrading RNA – even in small quantities. Laboratory personnel are the main source for RNase contamination as RNases are expressed in human keratinocytes and are present on skin and hairs.

- Separate the different procedures spatially.
  Ideally use separate rooms for sample preparation and PCR setup laid out to maintain a strict "one-way-system", thus avoiding cross-contamination in the work stream.
  At least dedicate different areas, equipment and consumables to each procedure.
- Establish a working area, designated as "RNase-free", in which only RNA work is performed. If the RNase-free working area is inside a lab with non-RNase-free working areas, clearly indicate RNase-free parts, e.g. using colour tape.
- Use dedicated RNase-free lab equipment (e.g. pipettes) for RNA-related work. Glassware has to be cleaned and decontaminated before use. For decontamination we recommend baking glassware at >200°C for ≥4 hours.
- Only use RNase-free tips and consumables which are guaranteed to be RNase-free.
- Control high risk areas for DNA / amplicon contamination on a regular basis (swabs / PCR analysis).
- Clean the real-time RT-PCR working area as described in the cleaning protocol (see section 2.4).
- Wear disposable gloves (latex or vinyl gloves) to prevent contamination with RNases which are present on human skin. Change gloves frequently during the procedure and / or after touching skin, hair, common surfaces etc.
- Wear a lab coat to prevent contamination from clothes.
- Always thaw RNA on a cooling block and store RNA at -20 °C or below.
- Handle real-time RT-PCR enzyme mix as briefly as possible at 0 °C or above. Do not mix reagents from different kits and do not mix reagents from different batches. Return all reagents to -20 °C after usage.
- Store VIR*Seek* kit components for real-time RT-PCR in dedicated areas, and apart from sample storage.
- Only open one tube at a time and always change pipette tips between liquid transfers to avoid cross-contamination.

2.4 Cleaning Protocol

Before commencing work and after completing the work, ensure that the real-time RT-PCR working area is cleaned as follows:

Cleaning steps	Cleaning protocol
1.	Decontaminate surfaces with Roti® Nucleic Acid-free* or 1 % HCl to remove DNA / RNA contamination.
2.	Clean the work surfaces and non-disposable laboratory equipment (pipettes, shaker, thermo shaker etc.) with an RNase decontaminating solution* (e.g. RNase AWAY®, Carl Roth, cat. no. A998) to remove RNase contaminations.

** Follow the manufacturer's instructions.*

2.5 Waste Disposal

Dispose of any waste which is potentially contaminated with a pathogenic virus according to your internal and local regulations.

For disposal of reagents and chemicals please refer to the material safety data sheet (MSDS).

2.6 Before you Begin

Store the cooling block for real-time RT-PCR at -20 °C overnight.

Extraction of viruses from different food matrices is performed according to the ISO 15216-1:2017 protocol.

For RNA extraction use suitable RNA extraction kits, for optimal performance Eurofins GeneScan Technologies' VIR*Seek* RNA*Extractor* Food kit (see section 1.3.1) is recommended.

2.7 Real-Time RT-PCR

2.7.1 Special Precautions during Real-Time RT-PCR Analysis

RT-PCR includes the reverse transcription (RT) of RNA into cDNA. RNA is a molecule which is particularly at risk of degradation due to abundant free RNases in the environment. Prior to RT, special emphasis has to be put on RNase-free environments (see section 2.3).

PCR is an exponential reaction. Therefore, after RT and amplification, the detection of single DNA targets is possible. The extreme sensitivity requires special precautions for handling and equipment. After a successful amplification, several billion amplicons are present in the reaction tube. Each of them might lead to a false positive result when contaminating sample material, i.e. by spreading as aerosols.

2.7.2 PCR Setup

Calculate required number of reactions and pipette all components (OligoMix and BasicMix) together and mix for the final reaction mix. The final real-time RT-PCR reaction mix is prepared with an additional 10 % volume.

Frequent freezing and thawing might cause inactivation of the reagents. Do not freeze / thaw kit components more than three times.

Components of final reaction mix	**Amount per reaction**	**e.g. for 10 real-time RT-PCR reactions (+ 10 %)**
BasicMix	5 µL	55 µL
OligoMix	10 µL	110 µL
Total volume	15 µL	165 µL

Before starting the practical working steps make sure you have switched on the computer, the PCR instrument and ensure the sample layout for the PCR plate is suitably documented and programmed (see below “Plate Setup”).

1. Place PCR plate or strips into the 96-well cooling block which has been cooled at -20 °C.
2. Add 15 µL of final reaction mix to each test well.
3. Add 5 µL Positive Control NoVGI, Negative Control and negative extraction control sample to the corresponding wells.
4. Add 5 µL of each sample to the corresponding reaction well of the PCR plate.
5. Use optical caps to seal the PCR plate / strips.
6. Spin down the plate / strips in a centrifuge.
7. Transfer the PCR plate / strips to the real-time RT-PCR instrument and start the run according to the thermocycler’s instructions.
8. Store samples at -20 °C or below in case PCR needs to be repeated.

Samples and Controls for Norovirus Genogroup I Assay

Designation	Volume of reaction mix	Addition of
Test samples	15 µL	5 µL of sample
Positive control (C^+)	15 µL	5 µL of Positive Control NoVGI
Negative extraction control (E^-)	15 µL	5 µL of negative extraction control sample
Negative control (C^-)	15 µL	5 µL of Negative Control

Plate Setup

The following PCR plate setup is recommended by Eurofins GeneScan Technologies GmbH if samples are only analysed for NoVGI and the process control virus. The controls correspond to the controls recommended by ISO 15216-1:2017.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C^+	S1					$(E^-)^1$	$(S1)^1$				
B	C^-	Sn					$(C^-)^1$	$(Sn)^1$				
C	E^-						$(PC^+)^1$					
D							$(PC\ 10^{-1})^1$					
E							$(PC\ 10^{-2})^1$					
F							$(PC\ 10^{-3})^1$					
G												
H												

[1] *Run with process control virus real-time RT-PCR Assay (e.g. VIRSeek Food Murine Norovirus (MNV) Process Control, refer to section 1.3.1)*

C^+:	positive control (of the target of interest)
C^-:	negative control
E^-:	negative extraction control
S1-Sn:	test samples
(PC^+):	process control
(PC (10^{-1}/10^{-2}/10^{-3})):	process control standard (10^{-1} / 10^{-2} / 10^{-3} dilution of RNA extraction from process control sample)

Thermal Profile

1 HOLD	1 HOLD	45 CYCLES	
reverse transcription	enzyme activation & reverse transcriptase inactivation	denaturation	annealing & extension
20 min at 50 °C	3 min at 95 °C	10 sec at 95 °C	60 sec at 60 °C
no data collection	no data collection	no data collection	data collection

For Bio-Rad CFX96 Touch™ Standard and Deep Well use default ramp rate.

Probe / Detection System

VIR*Seek* Food Norovirus Genogroup I	Fluorophore (Dye)
NoVGI	HEX™
IPC	Cy5™

3 DATA INTERPRETATION

Data is analysed manually by using the appropriate software provided by the cycler manufacturer.

Set baseline and threshold values according to manufacturers' instructions. It is recommended to use the automatic mode.

For the validated cyclers we recommend the following settings:

Real-time RT-PCR Thermocycler	Threshold	Baseline
Agilent AriaMX™	Auto	Adaptive
Bio-Rad CFX96 Touch™		Baseline Subtracted Curve Fit
Bio-Rad CFX96 Touch™ Deep Well		Baseline Subtracted Curve Fit

3.1 Export of Raw Data

For raw data export please follow the instruction in the corresponding cycler analysis software.

3.2 Evaluation of Results

The following tables provide an overview of the criteria to evaluate the run files:

Control evaluation

Control type	NoVGI	IPC	Overall results
Positive control (C^+)	22 ≤ Cq ≤ 38	Not relevant	Valid
	Cq < 22		Invalid*
	Cq > 38		Invalid*
Negative control (C^-)	No Cq	Cq ≤ 37	Valid
	No Cq	Cq > 37	Invalid*
	Cq ≠ "No Cq"	Not relevant	Invalid*

**Check amplification curve for sigmoid amplification signals, software background calculation and threshold settings*

Scoring of samples

Target name	Cq result	Target specific results
NoVGI	15 ≤ Cq ≤ 43	Positive
	Cq < 15	Check amplification curve for sigmoid amplification signals, software background calculation and threshold settings.
	Cq > 43	Compare to negative control. Check amplification, software background calculation and threshold settings.
	No Cq	Negative
IPC	Cq (C^-) -3 ≤ Cq Sample ≤ Cq (C^-) +3	Valid
	Cq Sample < Cq (C^-) -3	Unexpected result. Check amplification curve for sigmoid amplification signals, software background calculation and threshold settings.
	Cq Sample > Cq (C^-) +3	Sample inhibited.
	No Cq	Sample inhibited.

Final result interpretation for qualitative NoVGI real-time RT-PCR (including process control virus)

Preliminary sample result	IPC	Process control virus	Final results	Warning / measure
Positive for NoVGI	Not relevant	Not relevant	Positive for NoVGI	
Negative for NoVGI	Not inhibited	Valid	Negative for NoVGI	
Negative for NoVGI	Inhibited	Valid	Sample inhibited	Test 1:10 dilution of RNA extract of undiluted sample, see also ISO 15126-1:2017. If 1:10 dilution is still inhibited, repeat RNA extraction of the sample.
Negative for NoVGI	Inhibited	Invalid	Inhibited and extraction efficiency of process control virus is too low	Process control virus potentially inhibited, test 1:10 dilution of sample and process control virus. If 1:10 dilution is still inhibited, repeat RNA extraction of the sample. If process control virus is still invalid, repeat virus extraction.
Negative for NoVGI	Not inhibited	Invalid	Extraction efficiency of process control virus is too low	Repeat virus extraction

5 TROUBLESHOOTING

Observation:
No or weak fluorescence signal in HEX channel for the positive control

Possible Cause	Solution
Inappropriate storage of one or more kit components or kit is expired	Kit components have to be stored under appropriate conditions (see section 1.2) and only used up to the expiry date (printed on the kit label). Do not mix kit components from different lot numbers.
Kit components went through too many freeze-thaw cycles	Kit components (BasicMix, OligoMix) should not be freeze-thawed more than three times as it may cause inactivation of the reagents.
Selected channel does not conform to the protocol	Ensure that HEX channel has been chosen for analysis of the virus-specific amplification; select Cy5 channel for analysis of the internal positive control (IPC) amplification (see section 2.7.2).
Incorrect programming of the thermal profile	Check if thermal profile was defined according to the protocol (see section 2.7.2).
Incorrect real-time RT-PCR reaction mix setup	Real-time RT-PCR has to be repeated. Ensure that you use the correct volumes of OligoMix and BasicMix (see section 2.7.2).
Positive control not added to the test well	Make sure you added the positive control to the correct test well.

Observation:
No or weak Cy5 fluorescence signal of the internal control RNA (IPC)

Possible Cause	Solution
Inappropriate storage of one or more kit components, or kit components are expired	Kit components have to be stored under appropriate conditions (see section 1.2) and only used up to the expiry date (printed on the kit label).
Kit components went through many freeze-thaw cycles	Kit components (BasicMix, OligoMix) should not be freeze- thawed more than three times as it may cause inactivation of the reagents.
Real-time RT-PCR is inhibited	If fluorescence signal is only missed in one or more samples, but signals are detected in all of your controls, your sample/s may contain PCR-inhibitors. Common PCR-inhibitors are phenol, polysaccharides, detergents and compounds from source material (plant / animal tissue), which are carried-over from the sample preparation. Repeat real-time RT-PCR with a 1:10 dilution of your samples to examine if the inhibitory effect can be reduced. Make sure that you use an appropriate RNA isolation method, which removes potential real-time RT-PCR inhibitors. Ethanol as a common component of wash buffers has to be completely removed at the end of your RNA isolation.

To be continued

Possible Cause	**Cy5 channel has to be selected for analysis of the amplification of the internal positive control (IPC) (see section 2.7.2).**
Possible Cause	Check if thermal profile was defined according to the protocol (see section 2.7.2).
Possible Cause	Real-time RT-PCR has to be repeated. Ensure that you use the correct volumes of OligoMix and BasicMix (see section 2.7.2).

Observation:
Fluorescence signal in the HEX channel of the negative control (C^-)

Possible Cause	**Solution**
Contamination of your reactions	Real-time RT-PCR has to be repeated. If the C^- is negative in the repetition, the contamination occurred while loading the samples into the PCR reaction tubes. Only use RNA / DNA-free lab ware and consumables. Control all areas for DNA / amplicon contamination. Always clean the working area and equipment with Roti® Nucleic Acid-free or 1 % HCl to avoid DNA contamination from previous PCRs. PCR reaction tubes have to be closed immediately after loading the samples. If negative control C^- shows a fluorescence signal in the repetition, one or more of the kit components or the consumables (e.g. tubes) are contaminated. Use a new kit and observe Good Laboratory Practice (refer to EN ISO 22174 standard). We recommend using separate working areas for mix preparation, sample loading, and the real-time RT-PCR.

Observation:
No or weak fluorescence signal in HEX and / or Cy5 channel(s)

Possible Cause	**Solution**
Incorrect operation of the real-time PCR cycler	Check if your cycler operates according to the required specification. If necessary, perform cycler test, or contact the corresponding cycler service.

Observation:
Poor reproducibility between replicate samples

An increased variation in Cq-values across replicates can be due to poor pipetting technique or non-calibrated / imprecise pipettes. Comply with Good Laboratory Practice (refer to EN ISO 7218 standard) and check your equipment.

6 PRODUCT WARRANTIES, SATISFACTION GUARANTEE

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7 IMPORTANT NOTES

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TECHNICAL SUPPORT SERVICE

For technical assistance and more information please contact the Eurofins GeneScan Technologies GmbH Customer Service or your local distributor.

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