VIR*Seek* Murine Norovirus (MNV) Process Control Real-time RT-PCR

TEST KIT FOR MURINE NOROVIRUS (MNV)
QUANTITATIVE REAL-TIME RT-PCR FROM FOOD AND ENVIRONMENTAL SAMPLES

Cat. No. 5728200401

For 48 real-time RT-PCR reactions

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VIR*Seek*

Table of Contents

1 INTRODUCTION

The VIR*Seek* Murine Norovirus (MNV) Process Control Real-time RT-PCR kit provides materials for spiking of samples analysed for viruses and a subsequent quantification of the process control virus in order to control the virus isolation and pathogen detection process in food and surface samples. The kit has been validated for a diverse range of matrices including soft fruits, leaf, stem and bulb vegetables, bottled water, bivalve molluscan shellfish as well as on food surfaces, as described in the ISO 15216-2:2019 or environmental surfaces (WHO protocol "Surface sampling of coronavirus disease (COVID-19): A practical "how to" protocol for health care and public health professionals", Version: 1.1, 02/2020).

The kit contains the process control virus (only included in cat. no. 5728200401) and the corresponding real-time RT-PCR system and is intended to be used in analytical laboratories and may also be applied for other purposes in food product research and the analytical field, e.g. monitoring of contaminations during production processes.

The VIR*Seek* Murine Norovirus (MNV) Process Control 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 not intended for clinical diagnostics and should therefore be regarded as "For Research Use Only".

1.1 Background

According to ISO guidelines 15216-2:2019 the processes and horizontal methods for detection of hepatitis A virus (HAV) and norovirus (NoV) using real-time RT-PCR in food samples require the usage of a process control virus in order to monitor the quality of the entire viral extraction procedure.

Process control viruses need to have similar morphological and physicochemical properties as the target virus of interest.

MNV belongs to the family of *Caliciviridae* and is genetically related to the non-culturable human noroviruses. MNV is a culturable non-enveloped positive-sense single stranded RNA virus of a similar size to the target viruses. Therefore MNV can be considered a suitable process control virus for detection of HAV or human noroviruses for example.

1.2 Test Principle

During the analysis setup, a defined amount of the process control virus is spiked into the test sample at the earliest opportunity prior to virus extraction. In parallel a water sample that only contains the process control virus will be used as reference control for RNA extraction and real-time RT-PCR analysis.

After extraction of the viral RNA it is transcribed into cDNA during one-step real-time Reverse Transcriptase-PCR (RT-PCR). By means of specific primers nucleotide sequences of the species murine norovirus are amplified during the following PCR.

DNA amplification and detection take advantage of the nucleotide sequence conservation found in viral genomes that ensures the potential for high specificity and sensitivity in detection of the murine norovirus. Primers do not cross-react with transcribed DNA from other common food-borne virus species including norovirus, hepatitis A virus, hepatitis E virus, rotavirus, and also not with DNA derived from other relevant biological material.

By monitoring the extraction of the process control virus the efficiency of the virus testing protocol for food samples like soft fruits, leaf-, stem- and bulb- vegetables, bottled water, bivalve molluscan shellfish, as well as food and environmental surfaces can be analysed.

1.3 Components of the Kit

For real-time RT-PCR: Cat. No. **5728200401**

- 1x **Oligo Mix MNV**[*], vial with purple cap with primers and probe for MNV RNA, 530 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 3 times.
- 1x **Basic Mix**[*] **VIR*Seek***, vial with white cap, 265 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 3 times.
- 1x **Murine Norovirus**[*#] (10^8 copies / mL) 1 vial with transparent cap, 500 µL, store at -20 °C ± 2 °C [1)], do not freeze / thaw more than 3 times.
- 1x **Negative Control**, vial with transparent cap, 500 µL, store at -20 °C ± 2 °C.
- 1x **Molecular grade water**, white bottle, 30 mL, store at room temperature.

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

[#] *Shipped on dry ice in rigid secondary packaging separately from the other kit components (according to UN 3373 and packaging instruction p650).*

[1)] *Best storage temperature for virus stability is at -80°C.*

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- 1x **Oligo Mix MNV**[*], vial with purple cap with primers and probe for MNV RNA, 530 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 3 times.
- 1x **Basic Mix**[*] **VIR*Seek***, vial with white cap, 265 µL, store at -20 °C ± 2 °C, do not freeze / thaw more than 3 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.*

1.4 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. Mini Centrifuge IKA Mini G, cat. no. 5613902601 or VWR, MiniStar silverline cat. no. 521-2844P).
  - 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.

1.4.1 Complete Portfolio of the VIR*Seek* 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 Hepatitis E Virus**, cat. no. 5728200501
  - Real-time RT-PCR kit with 48 reactions for rapid detection of hepatitis E virus (HEV) 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* SARS-COV-2 Screen**, cat. no. 5728200601
  - Real-time RT-PCR kit with 96 reactions for rapid screening for SARS-CoV-2 E-gene sequence in environmental and food surface samples.
- **VIR*Seek* SARS-CoV-2 Ident**, cat. no. 5728200701
  - Real-time RT-PCR kit with 96 reactions for rapid detection of SARS-CoV-2 specific RdRP sequence in environmental and food surface samples.
- **VIR*Seek* Murine Norovirus (MNV) Process Control**, cat. no. 5728200401
  - Murine Norovirus spiking material (0.5 mL) and real-time RT-PCR kit with 48 reactions for rapid detection of murine norovirus (MNV) process control virus in food and environmental samples.
- **VIR*Seek* Murine Norovirus (MNV)**, cat. no. 5728200801
  - Real-time RT-PCR kit with 48 reactions for rapid detection of murine norovirus (MNV) in food, environmental and food surface samples.
- **VIR*Seek* RNA*Extractor***, cat. no. 5524400101
  - Kit for extraction of viral RNA via silica-coated magnetic beads from different samples. 48 x 500 µL or 96 x 250 µL sample input volume or 96 x surface samples.
- **VIR*Seek* RNAExtractor Magnetic Beads**, cat. no. 5524400501
  - For 48 x 500 µL or 96 x 250 µL sample input volume or 96 x surface samples.
- **VIR*Seek* RNAExtractor Carrier Molecule**, cat. no. 5524400601
  - For 48 x 500 µL or 96 x 250 µL sample input volume or 96 x surface samples.
- **VIR*Seek* PathoSwab 50**, cat. no. 5728200901
  - Kit for 50 virus sample acquisitions. All items/reagents provided.

2 HOW TO USE THIS PRODUCT

2.1 Important Notes:

- Store all reagents as indicated in section 1.3.
- 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 is performed according to the ISO 15216-2:2019 and WHO protocol (see 1).
- Aliquot murine norovirus during first usage if frequently small sample numbers are processed.
- Avoid keeping the aliquot at 4°C or room temperature.
- For RNA extraction use suitable RNA extraction kits, for optimal performance we recommend to use the Eurofins GeneScan Technologies VIR*Seek* RNA*Extractor* kit.

2.2 General and Safety Precautions

- All samples should be handled with caution as they are potentially infectious.
- The VIR*Seek* Murine Norovirus (MNV) Process Control Real-time RT-PCR kit contains murine norovirus, which is a hazardous good and is assigned to UN3373 “Biological substances, Category B”. Use the product only in accordance with the regional / national biosafety regulations. For more information, please refer to the VIR*Seek* kits MSDS.
- The VIR*Seek* Murine Norovirus (MNV) (Process Control) Real-time RT-PCR kit contains glycerol and propane-1,2-diol which may cause mild skin irritation.

2.3 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 15216-2:2019 for HAV and norovirus detection in food samples or WHO guideline (see 1) for SARS-COV-2 in environmental surface samples.
- Perform cleaning protocol (outlined in section 2.5).
- Use DNA-, nuclease-free, and sterile lab ware.
- Wear gloves and change frequently.

2.4 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 takes place. If the RNase-free working area is inside a lab with none RNase-free working areas, clearly indicate RNase-free parts, e.g. using color 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 2.5).
- 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.
- Avoid breathing on samples or working area: Wear a disposable face mask or protect the designated RNase-free working area with a screen.
- Always thaw RNA on a cooling block and store RNA at -20 °C or below.
- Handle real-time RT-PCR enzyme mix as short 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.

2.5 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.*

2.6 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).

2.7 Before you Begin

Please note: If you are using the cat. no. 5728200801, skip to section 2.8.

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

2.7.1 Preparation of Samples

MNV is added in a defined amount to the test samples prior to virus extraction in order to monitor the quality of the entire viral extraction procedure (recovery rate).

Therefore, defrost a sufficient volume of murine norovirus (10 µL x number of samples to be analysed). Vortex MNV and spin down shortly. Add 10 µL of MNV to each sample (for swabs: add 10 µL MNV directly on the swab tip) and perform virus extraction as described in the ISO 15216-2:2019 and WHO protocol (see 1). Use isolated virus directly for RNA extraction or store at -20 °C or below (< 6 months), respectively.

For RNA extraction use suitable RNA extraction kits, for optimal performance we recommend to use Eurofins GeneScan Technologies' VIR*Seek* RNA*Extractor* kit (see section 1.4.1).

2.7.2 Preparation of Process Control

To determine the recovery rate of the test samples, add 10 µL of MNV to a separate portion of water (process control; volume depending on RNA extraction protocol applied). Viral RNA extraction of the test samples and the process control is performed in parallel with the same extraction method. Use isolated RNA directly for real-time RT-PCR or store at -20 °C or below (<6 months), respectively.

2.7.3 Preparation of Process Control Standard

In order to quantify the extracted process control virus, a standard curve should to be prepared.

Prepare three times a 1:10 dilution (in molecular grade water) of the RNA extract from the process control (10^{-1}, 10^{-2} and 10^{-3}).

The standard should always be prepared fresh directly before real-time RT-PCR setup.

2.8 Real-Time RT-PCR

2.8.1 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 2.4).

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.

2.8.2 PCR Setup

Calculate required number of reactions and pipette all components (Oligo Mix and Basic Mix) 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 %)**
Basic Mix	5 µL	55 µL
Oligo Mix	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 PC^{+}, PC 10^{-1}/ 10^{-2}/ 10^{-3}, Negative Control, negative extraction control sample and/ or negative sampling control and negative sampling device control (surface samples) to the corresponding well.

4. Add 5 µL of each sample in 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 thermo cycler manufacturer instructions.
8. Store samples at -20 °C or below in case of PCR needs to be repeated.

Samples and Controls for Process Control Virus Assay

Designation	Volume of reaction mix	Addition of
Test samples	15 µL	5 µL of sample
Process control (PC^+)	15 µL	5 µL of RNA extraction from process control sample (see ISO 15216-1:2017)
Process control standard (PC 10^{-1}/ 10^{-2}/ 10^{-3})	15 µL	5 µL of each dilution (10^{-1}/ 10^{-2}/ 10^{-3}) of RNA extraction from process control sample
Negative extraction control (E^-)	15 µL	5 µL of negative extraction control sample
Negative control (C^-)	15 µL	5 µL Negative Control
Negative sampling control (S^-)	15 µL	5 µL of negative sampling control sample
Negative sampling device control (SD^-)	15 µL	5 µL of negative sampling device control sample

Plate Setup

The following PCR plate setup is recommended by Eurofins GeneScan Technologies GmbH if samples are only analysed for one target virus and the process control virus. The controls correspond to the controls recommended by ISO 15216-2:2019 and WHO protocol (see 1).

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

[1] *Run with target virus real-time RT-PCR Assay*

[2] *ISO 15216-2:2019 recommends testing samples in PCR duplicates*

** The respective WHO guideline (see 1.2.3) recommends including negative swab and swabbing device samples.*

- (C^+): positive control (of the target of interest)
- C^-: negative control
- E^-: negative extraction control
- S^-: negative sampling control
- SD^-: negative sampling device control
- S1.1 – Sn.2: test samples in duplicates
- 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

Two thermal profiles have been validated in order to ensure the compatibility of the VIR*Seek* Murine Norovirus (MNV) Process Control Real-time RT-PCR kit with the VIR*Seek* Food product line, as well as for the VIR*Seek* SARS-CoV-2 solution.

Thermal Profile 1 (adapted to VIR*Seek* Food product line):

1 HOLD	**1 HOLD**	**45 CYCLES**[1)]	
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.

[1)] For Bio-Rad CFX96 Touch™ Standard and Deep Well the number of cycles is set to 44 to carry out 45 cycles.

Thermal Profile 2 (adapted to VIR*Seek* SARS-CoV-2 solution):

1 HOLD	**1 HOLD**	**40 CYCLES**[1)]	
reverse transcription	enzyme activation & reverse transcriptase inactivation	denaturation	annealing & extension
10 min at 50 °C	3 min at 95 °C	3 sec at 95 °C	30 sec at 58 °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.

[1)] For Bio-Rad CFX96 Touch™ Standard and Deep Well the number of cycles is set to 39 to carry out 40 cycles.

Probe / Detection System

VIR*Seek* Murine Norovirus (MNV) Process Control	**Fluorophore (Dye)**
MNV	FAM™

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[1)]	Adaptive
Bio-Rad CFX96 Touch™	Auto	Baseline Subtracted Curve Fit[2)]
Bio-Rad CFX96 Touch™ Deep Well		Baseline Subtracted Curve Fit[2)]

1) If appropriate, auto calculated threshold with default background based threshold settings can be used: Cycle range: 5 thru 9; Sigma multiplier: 10.

2) Always apply fluorescence drift correction

3.1 Export of Raw Data

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

3.2 Process Control Virus Recovery

(According to ISO 15216-2:2019)

a. A dilution series of an RNA extraction of the process control virus (PC^+, PC 10^{-1}/ 10^{-2}/ 10^{-3}) in RNase-free water should be used as quantification standard.

b. The standard curve is acceptable if R^2 value > 0.98 and slope is between |3.1| und |3.6| (corresponding to an amplification efficiency between 90 % and 110 %).

c. One outlier value can be removed according to ISO 15216.

d. The sample analysis is valid if the process control virus recovery is ≥ 1 % (see below), otherwise the virus extraction must be repeated.

Note: Samples are in general analysed undiluted. In case of extraction efficiency less than 1 % in a sample, the sample needs to be repeated 1:10 diluted (in water). Extraction efficiency < 1 % may be due to inhibitors present in the sample. To check for these inhibitors a dilution as recommended by the ISO 15216-2:2019 is performed. If the recovery rate of the measured value multiplied with the dilution factor is ≥ 1 %, the sample is considered valid.

Process control virus recovery (P):

$$P\,[\%] = 10^{(\Delta Cq/m)} \times 100\%\ (\times D)$$

ΔCq = Cq value [sample RNA] – Cq value [undiluted process control virus RNA]

m = slope of the standard curve

D = Dilution factor (= 10, in case of a 1:10 dilution of the sample)

For bivalve molluscan shellfish (BMS):

Extraction efficiency $[\%] = \left(\frac{P}{0.5}\right) \times V$

P = Process control virus recovery in %

V = Total volume of supernatant in mL

For all other matrices (where total volumes of virus extract are used):

Extraction efficiency $[\%] = P$

Control evaluation

Thermal Profile 1 (adapted to VIR*Seek* Food product line):

Negative Control	Status
Positive (Cq ≤ 40)	Invalid Samples shall be retested
Negative (Cq > 40)	Valid*

** Cq values > 40 have no significant influence on process control virus recovery quantification results.*

Thermal Profile 2 (adapted to VIR*Seek* SARS-COV-2 solution):

Negative Control	Status
Positive (Cq < 40)	Invalid Samples shall be retested
Negative (No Cq)	Valid*

4 TROUBLESHOOTING

Observation:
No or weak fluorescence signal in FAM channel for the process control and/or samples

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 1.3) and only used up to the date of expiry (printed on the kit label). Do not mix kit components of different lot numbers.
Kit components passed too many freeze thaw cycles	Kit components (Basic Mix, Oligo Mix) should not be thawed more than three times as it may cause inactivation of the reagents.
Selected channel do not conform to the protocol	Ensure that FAM channel has been chosen for analysis of the virus-specific amplification (see section 2.8.2).
Incorrect programming of the thermal profile	Check if thermal profile was defined according to the protocol (see section 2.8.2).
Incorrect real-time RT-PCR reaction mix setup	Real-time RT-PCR has to be repeated. Ensure that you use the correct volumes of Oligo Mix and Basic Mix (see section 2.8.2).
Instability of virus at room temperature and at 4 °C	Make sure that you do not store the virus at elevated temperature. Start directly your workflow after thawing.
MNV not added to the RNA extraction process control / samples	Make sure that you added MNV before RNA extraction.
Inappropriate method for RNA isolation	When signal is missed in all of the samples and the process control, ensure that you use an appropriate RNA isolation method. No signal can be due to insufficient virus lysis or RNA loss while RNA isolation.
Real-time RT-PCR is inhibited	If fluorescence signal is only missed in one or more samples, but signal is simultaneously present in the process control, your sample/s may contain PCR- inhibitors. Common PCR- inhibitors are phenol, polysaccharides, detergents and compounds 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	Solution
Isolated RNA is degraded due to RNase contamination	To prevent RNase contamination wear a lab coat and suitable gloves, which should be changed frequently during the procedure. Only use RNase-free pipette tips. Decontaminate work surfaces and non-disposable laboratory equipment (pipettes, vortex mixer) with appropriate reagents.
RNA degradation during sample storage	Real-time RT-PCR should be done immediately after RNA isolation. Otherwise ensure that samples are stored suitably (-20 °C (<6 months), -80 °C (>6 months)).
Isolated RNA is degraded due to freeze-thaw cycles	Minimize freeze- thaw cycles of the samples to avoid RNA degradation.
Incorrect operation of the real-time PCR cycler	Control if your cycler operates according to the required specification. If needed perform cycler test, or contact the corresponding cycler service.

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

Possible Cause	Solution
Contamination of your reactions	Real-time RT-PCR has to be repeated if Cq ≤ 40 Values above 40 have no significant influence on the process control virus recovery quantification results. Important: Low level contaminations with MNV indicate that cross-contamination happened in the lab and processes should be reviewed thoroughly! If the C^- is valid in the repetition, the contamination occurred while loading the samples into the PCR reaction tubes. Only use DNA / RNA-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 again a fluorescence signal below Cq 40 in the repetition, one or more of the kit components or the consumables (e.g. tubes) are contaminated. Use a new kit and observe the 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 performance.

Observation:
Fluorescence signal in the FAM channel of the negative extraction control (E^-)

Possible Cause	Solution
Contamination of your reactions	Real-time RT-PCR has to be repeated if Cq ≤ 40. If the E^- is valid in the repetition, the contamination occurred while loading the samples into the PCR reaction tubes. Always clean the working area and equipment with Roti® Nucleic Acid-free or 1 % HCl to avoid contamination from previous PCRs. PCR reaction tubes have to be closed immediately after loading the samples. If negative extraction control E^- shows again a positive signal in the repetition, but C^- is simultaneously negative, contamination occurred while RNA extraction. To avoid cross contamination while performing RNA extraction, follow the Good Laboratory Practice. If negative extraction control E^- shows again a positive signal in the repetition and signal is simultaneously present in the negative control (C^-), one or more of the kit components or the consumables (e.g. tubes) are contaminated. Use a new kit and observe the 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 performance.

Observation:
Poor reproducibility between replicate samples

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

Observation:
Poor PCR efficiency or correlation coefficient of the standard curve

PCR efficiency should be between 90 % and 110 % (corresponding to a slope between |3.1| and |3.6| of the standard curve) and R^2 should be > 0.98. PCR efficiency below |3.1| or R^2 ≤ 0.98 can be due to poor pipetting techniques or non-calibrated/ imprecise pipets and cyclers. Comply with the Good Laboratory Practice (refer to EN ISO 7218 standard) and check your equipment.

5 APPENDIX

5.1 Validation Data Sheet

See in the corresponding validation data sheet.

6 PRODUCT WARRANTIES, SATISFACTION GUARANTEE

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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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