Challenge

Sensitive detection of various targets in wastewater-based epidemiology (WBE); these include pathogens and human fecal markers that indicate the presence of human material in a sample.

Solution

Detection of pathogenic norovirus genogroups I/II and the fecal markers Bacteroides HF183, CrAssphage, and PMMoV by application of the Analytik Jena workflow for biosurveillance in wastewater with the RealStar® Norovirus RT-PCR Kit 3.0 from Altona Diagnostics and self-developed assays.

Monitoring Wastewater for Epidemiologically Relevant Targets via Real-time PCR

Introduction

As efforts are made to mitigate effects of the SARS-CoV-2 pandemic by monitoring the prevalence of this virus in wastewater, there is an increasing interest in additional information that might be obtained from sewage: The qPCR-based detection of specific target genes can be used to detect pathogens circulating in the population, and reference genes can be utilized as markers of human material in these samples. Here, various targets such as the human-pathogenic norovirus (genogroups I and II) and three human fecal markers, namely Cross-assembly phage (CrAssphage), bacteroid HF183 and Pepper mild mottle virus (PMMoV), were analyzed. CrAssphage and HF183 are both DNA targets which are present in the human intestine and thus within excretions. PMMoV is an RNA target. It is globally prevalent in pepper crops, its presence in human feces is thus diet-related. Noroviruses are highly infectious, fecal-oral transmissible RNA viruses. They cause large outbreaks of gastroenteritis, e.g., in hospitals, nursing homes, or on cruise ships. Since genogroups I and II are not or hardly detectable by classical methods, PCR assays are the tool of choice for diagnostic testing. To detect local outbreaks, examining wastewater from a specific sampling point of the sewage system is useful. Thus, disease management can be restricted to a localized group of individuals while maintaining anonymity. Through subsequent precautions, a major outbreak can be prevented. Due to the unknown but possibly high content of PCR inhibitors, wastewater is a challenging sample matrix which requires high-quality analytical processes.

Analytik Jena provides a complete workflow that enables the detection of target genes like norovirus genogroup I and II, HF183, CrAssphage, and PMMoV in wastewater. First, nucleic acids are concentrated by filtration. This is followed by purification steps, including extraction of the filter using the homogenizer SpeedMill PLUS (Analytik Jena), and automated nucleic acid extraction on the

InnuPure C16 *touch* (Analytik Jena) in combination with the innuPREP AniPath DNA/RNA Kit (IST Innuscreen GmbH). The detection of the target genes is performed on the quantitative real-time PCR device qTOWER³ (Analytik Jena) by amplification of target sequences with the respective qPCR assays.
The workflow process is simple and effective: Filtration is a fast and cost-efficient method for concentration, and automated nucleic acid extraction with the InnuPure C16 *touch* is reliable and easy-to-handle. The qTOWER³ guarantees a sensitive detection with multiplex PCR option for parallel determination of multiple targets. Due to the challenging sample matrix, PCR assays have to be optimized and tested for the use with wastewater samples. The norovirus detection kit from Altona Diagnostics was shown to meet those requirements. For the other markers, own assays were developed, a single PCR detection of PMMoV, and a duplex detection of HF183 and CrAssphage, respectively.

Materials and Methods

Samples, Reagents, and Consumables

- innuSPEED Lysis Tubes J (845-CS-1120100, IST Innuscreen GmbH)
- innuPREP AniPath DNA/RNA Kit - IPC16 (845-IPP-8016096 or 845-PPP-8016096, IST Innuscreen GmbH)
- MF-Millipore MCE Membrane Filter, hydrophilic, 0.45 µm, Ø 47 mm (HAWP04700, Merck Millipore)
- 96 well microplate, full skirted, white (844-70038-S, Analytik Jena GmbH)
- Optical Sealingfoil (844-70046-0, Analytik Jena GmbH)
- DNA/RNA-Shield (R1100, Zymo-Research)
- RealStar® Norovirus RT-PCR Kit 3.0 (053003, Altona Diagnostics)
- SensiFAST Probe No-ROX Kit (BIO-86005, Meridian)
- Tetro cDNA Synthesis Kit (BIO-65042, Meridian)
- Primers and Probe for HF183 (biomers.net GmbH)
  Primer fwd.: ATC ATG AGT TCA CAT GTC CG,
  Primer rev.: CTT CCT CTC AGA ACC CCT ATC C,
  Probe: 6-FAM-CTA ATG GAA CGC ATC CC-BMN-Q535
- Primers and Probe for CrAssphage (biomers.net GmbH)
  Primer fwd.: CAG AAG TAC AAA CTC CTA AAA AAC GTA GAG,
  Primer rev.: GAT GAC CAA TAA ACA AGC CAT TAG C,
  Probe: Cy5-AAT AAC GAT TTA CGT GAT GTA AC-BMN-Q620
- Primers and Probe for PMMoV (biomers.net GmbH)
  Primer fwd.: GAG TGG TTT GAC CTT AAC GTT GA,
  Primer rev.: TTG TCG GTT GCA ATG CAA GT,
  Probe: 6-FAM-CCT ACC GAA GCA AAT G-MGB-CDPI3
- 500 mL wastewater (from wastewater treatment plant influent)

Instruments

- Stainless steel pressure filter holder (16249, Sartorius) and tubing/fixation
- Compressor/inflator (battery-driven, 8 bar pressure, for instance Makita DMP180Z)
- SpeedMill PLUS (Analytik Jena GmbH; 230V grid: 845-00007-2; 110V grid: 845-00007-4)
- InnuPure C16 *touch* (845-00020-2, Analytik Jena GmbH)
- qTOWER³ qPCR device (Analytik Jena GmbH) in appropriate system configuration:
  Basic unit with color module 1 to detect FAM (230V: 844-00553-2; 115V: 844-00553-4; 100V: 844-00553-5)
  Color module 2 for JOE detection (844-00521-0)
  Color module 5 for Cy5 detection (844-00524-0)
- Centrifuge (for 2 mL tubes)
- Vortex mixer
- Pipettes
- Forceps

Methods

1. Concentration and purification of wastewater samples

The wastewater sample was taken and split into five equal parts. These were concentrated by filtration as described in the application note "Complete PCR-based Detection Workflow of SARS-CoV-2 in Wastewater"[1] with the exception that DNA/RNA-Shield instead of PBS was added to the membranes after filtration. The DNA/RNA Shield medium stabilizes nucleic acids at ambient temperatures. This methodological improvement allows samples to be transported to the analytical laboratory without refrigeration and to be stored at 4 °C for up to 6 days. As previously described[1], filter-adsorbed molecules were released using beaded tubes and the SpeedMill PLUS. Subsequently, nucleic acids were extracted with the InnuPure C16 *touch* using the innuPREP AniPath DNA/RNA Kit (Protocol 2: "Isolation from 400 µL cell-free body fluids, cell culture supernatants and whole blood", elution volume 100 µL). After extraction, samples were directly processed or stored at -20 °C, respectively.

2 A. Detection of norovirus genogroups I and II

The norovirus detection was performed using the RealStar® Norovirus RT-PCR Kit 3.0 following the manufacturer's instructions. The samples were analyzed in duplicate, the Internal Control was added to the Master Mix and used as RT-PCR inhibition control. PCR protocol setup within the qPCRsoft was done according to the kit instructions (Figure 1 and 2). Norovirus genogroup I was measured in the Cy5 channel (color module 5), norovirus genogroup II in the FAM channel (color module 1), and the Internal Control was detected in the JOE channel (color module 2).

2 B. Determination of CrAssphage and HF183 in a duplex assay

Quantitative PCR was carried out using a procedure previously published[2]. The samples were analyzed in duplicate. Each reaction contained 10 µL SensiFAST Probe No-ROX Master Mix, 600 nM of primers specific for HF183, 400 nM of specific CrAssphage primers, 200 nM of each probe, and 5 µL of template in a total volume of 20 µL. PCR-grade water was used for volume adjustments and as an NTC. The temperature profile and the dye acquisition were set as shown in Figure 3 and 4. The FAM-labeled probe for determination of HF183 was measured in channel 1 and the Cy5-labeled probe for CrAssphage in channel 5 of the qTOWER³.

Lid temp. °C: 100 Preheat lid Device: qTOWER³G

	5 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		55,0	20:00	--	---	--,-	---	8,0
	2		95,0	02:00	--	---	--,-	---	8,0
45x	3		95,0	00:15	--	---	--,-	---	8,0
	4	◆	55,0	00:45	--	---	--,-	---	6,0
	5		72,0	00:15	3	44	--,-	---	6,0
	6								
	7								
	8								
	9								
	10								

Figure 1: Temperature profile applied on qTOWER³ (Analytik Jena) for RealStar® Norovirus RT-PCR Kit 3.0 (Altona Diagnostics).
Step 1 – Reverse transcription,
Step 2 – Initial denaturation,
Step 3 + 4 + 5 – Amplification (Denaturation + Annealing + Elongation), data acquisition is done in step 4.

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	JOE	5	◆	
3	Yellow	TAMRA	5		
4	Orange	ROX	5		
5	Red	Cy5	5	◆	
6	NIR1	Cy5.5	5		

Meas. repeats: 3 Color compensation: Standard1

Scan region according layout
Define scan region manually

Figure 2: Scan settings on qTOWER³ for RealStar® Norovirus RT-PCR Kit 3.0 (Altona Diagnostics).

Lid temp. °C: 100 Preheat lid Device: qTOWER³G

	3 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		95,0	10:00	--	---	--,-	---	8,0
40x	2		95,0	00:15	--	---	--,-	---	8,0
	3	◆	60,0	01:00	2	39	--,-	---	6,0
	4								
	5								
	6								
	7								
	8								
	9								
	10								

Figure 3: Temperature profile for the HF183/CrAssphage duplex assay on qTOWER³ (Analytik Jena).
Step 1 – Initial denaturation,
Step 2 + 3 – Amplification (Denaturation + Elongation), data acquisition is done in step 3.

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	JOE	5		
3	Yellow	TAMRA	5		
4	Orange	ROX	5		
5	Red	Cy5	5	◆	
6	NIR1	Cy5.5	5		

Meas. repeats: 3 Color compensation: Aus

Scan region according layout
Define scan region manually

Figure 4: Scan settings for the HF183-CrAssphage duplex assay on qTOWER³.

2 C. Determination of PMMoV

Reverse transcription of PMMoV RNA was carried out with the Tetro cDNA Synthesis Kit according to the kit manufacturer's instructions. A maximum of 0.7 µg RNA was used as template. PCR-grade water was used as the NTC. The reaction was processed on the qTOWER^3, with the temperature profile shown in Figure 5.

The qPCR protocol used a modification of a method published elsewhere[3]. The samples were tested in duplicate. Each reaction unit contained 10 µL SensiFAST Probe No-ROX Master Mix, 500 nM of primers specific for PMMoV and 125 nM of the probe. 3 µL of cDNA sample or PCR grade water as NTC was added. The reaction mixture was made up to 20 µL with PCR grade water. The temperature profile and dye acquisition were adapted from the publication (Figure 6 and 7). The FAM-labeled probe was detected via channel 1 of the qTOWER^3.

Lid temp. °C: 100 Preheat lid Device: qTOWER³

3 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
1		25,0	10:00	--	---	--,-	---	8,0
2		45,0	30:00	--	---	--,-	---	6,0
3		85,0	05:00	--	---	--,-	---	6,0
4								
5								
6								
7								
8								
9								
10								

Figure 5: Temperature profile for PMMoV cDNA synthesis on qTOWER^3.

Lid temp. °C: 100 Preheat lid Device: qTOWER³G

3 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
45x [1		95,0	10:00	--	---	--,-	---	8,0
2		95,0	00:10	--	---	--,-	---	8,0
3	◆	60,0	00:30	2	44	--,-	---	6,0
4								
5								
6								
7								
8								
9								
10								

Figure 6: Temperature profile for the PMMoV assay on qTOWER^3. Step 1 – Initial denaturation, Step 2 + 3 – Amplification (Denaturation + Elongation), data acquisition is done in step 3.

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	JOE	5		
3	Yellow	TAMRA	5		
4	Orange	ROX	5		
5	Red	Cy5	5		
6	NIR1	Cy5.5	5		

Meas. repeats: 3 Color compensation: Aus

Scan region according layout

Define scan region manually

Figure 7: Scan settings for the PMMoV assay on qTOWER^3.

Results and Discussion

A: Detection of Norovirus Genogroups I and II

The PCR run is validated by the positive genogroup controls, the negative NTC (Figure 8 and 9, Table 1 and 2), and the successful amplification of the internal control (Figure 10, Table 3). Virus detection was successful, the tested wastewater sample was positive for norovirus genogroups I and II.

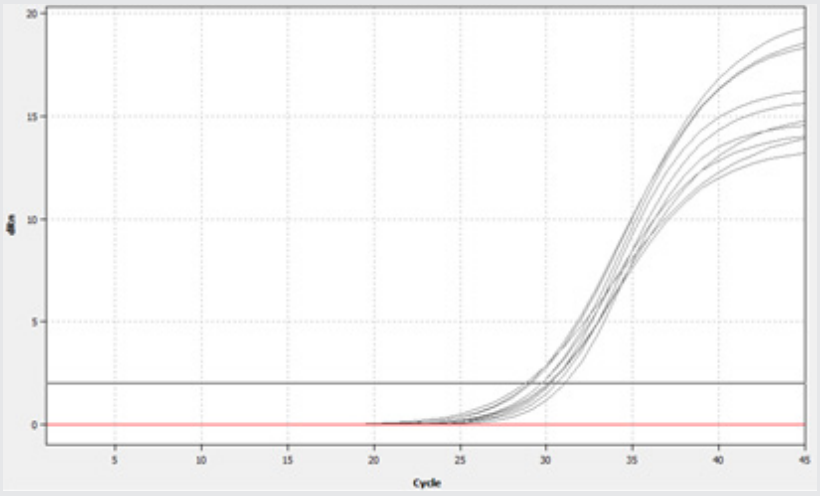

Figure 8: Amplification curves of the Cy5-labeled Norovirus genogroup I target sequence. Five sample aliquots, analyzed in duplicate (grey), are shown as well as the NTC (red).

Table 1: Ct values of Norovirus genogroup I target gene amplification curves.

Sample aliquot	Mean Ct
A	29.48
B	30.20
C	31.14
D	30.25
E	29.40
PC Genogroup I	29.69
NTC	No Ct

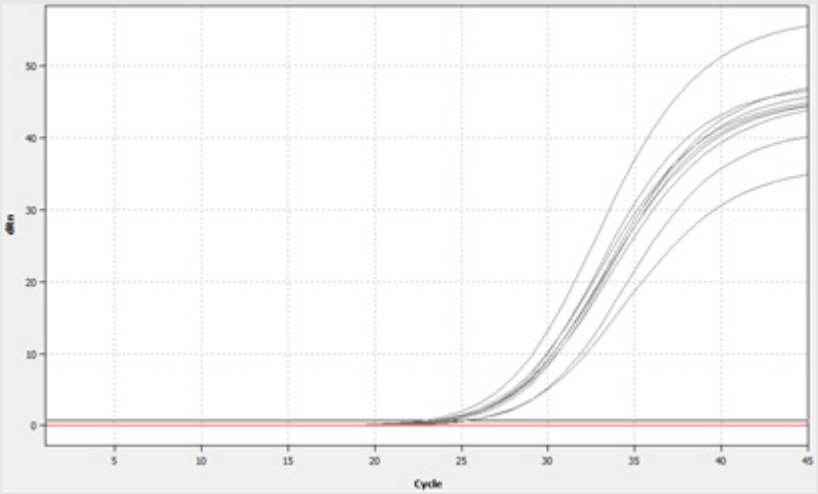

Figure 9: Amplification curves of the FAM-labeled Norovirus genogroup II target sequence. Five sample aliquots, analyzed in duplicate (grey), are shown as well as the NTC (red).

Table 2: Ct values of Norovirus genogroup II target gene amplification curves.

Sample aliquot	Mean Ct
A	24.61
B	23.61
C	23.65
D	23.89
E	23.73
PC Genogroup II	29.27
NTC	No Ct

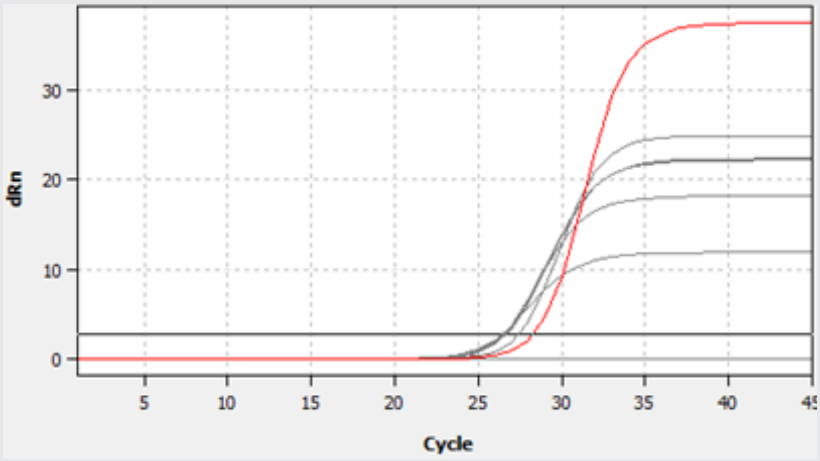

Figure 10: Amplification curves of the JOE-labeled Internal Control target sequence. Shown are five sample aliquots analyzed in duplicate (grey) and the NTC (red).

Table 3: Ct values of Internal Control target gene amplification curves.

Sample aliquot	Mean Ct
A	27.39
B	26.40
C	26.55
D	26.46
E	26.61
NTC	28.25

B: Detection of CrAssphage and HF183 in a Duplex Assay

This PCR run is valid, because the NTC is negative. Since this is a self-assembled assay no positive controls have been included in the PCR setup. As both targets were detected in every sample, the positive control was not necessary to confirm the functionality of the test.

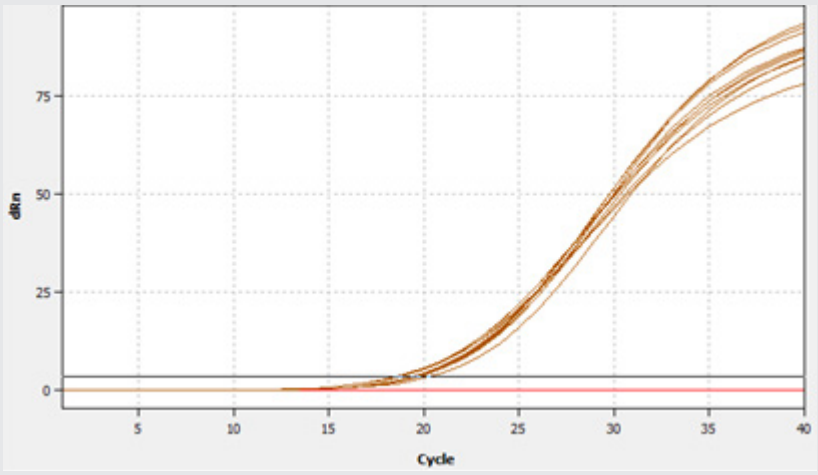

Figure 11: Amplification curves of the FAM-labeled HF183 target sequence. Five sample aliquots, analyzed in duplicate (brown), are shown as well as the NTC (red).

Table 4: Ct values of HF183 target gene amplification curves.

Sample aliquot	Mean Ct
A	18.72
B	20.06
C	19.56
D	19.48
E	18.78
NTC	No Ct

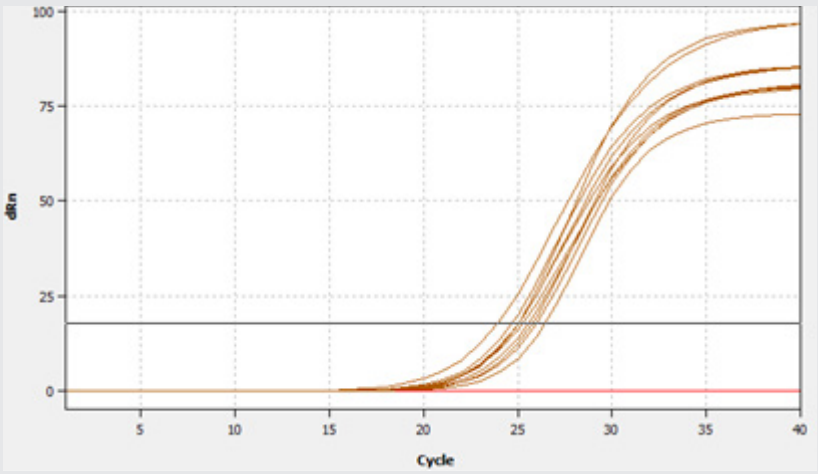

Figure 12: Amplification curves of the Cy5-labeled CrAssphage target sequence. Five sample aliquots, analyzed in duplicate (brown), are shown as well as the NTC (red).

Table 5: Ct values of CrAssphage target gene amplification curves.

Sample aliquot	Mean Ct
A	24.58
B	26.09
C	25.30
D	25.50
E	24.84
NTC	No Ct

C: Detection of PMMoV

PCR assay validity is shown by the negative NTC of the PCR run and the negative NTC of the cDNA synthesis. All samples show an amplification curve, thus the wastewater samples contained the PMMoV target sequence.

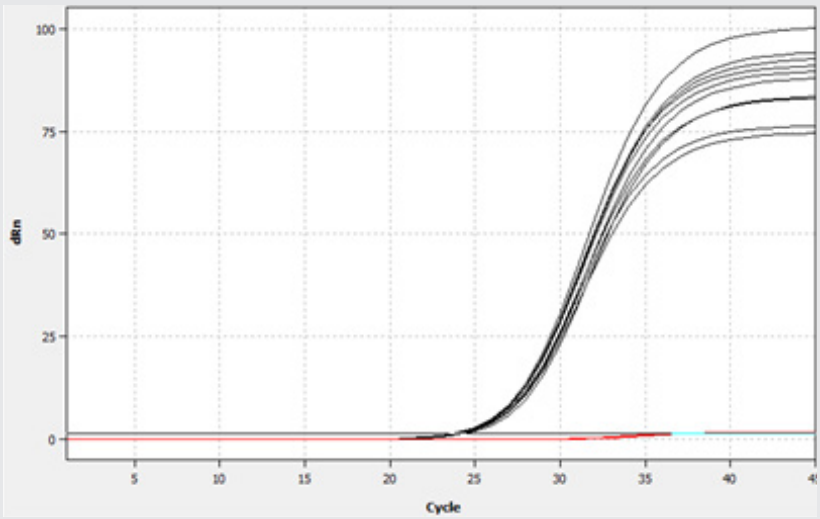

Figure 13: Amplification curves of the FAM-labeled PMMoV target sequence. Five sample aliquots, analyzed in duplicate are shown (black), in addition the NTC and the NTC of cDNA synthesis (red).

Table 6: Ct values of PMMoV target gene amplification curves.

Sample aliquot	Mean Ct
A	24.16
B	23.92
C	23.92
D	24.10
E	24.33
NTC cDNA synthesis	No Ct
NTC	No Ct

Overview of results

The RealStar® Norovirus RT-PCR Kit 3.0 as well as the two assays developed for HF183/CrAssphage and PMMoV were successfully applied to detect their target sequences (Figure 14). In addition, the assay consistencies were very good, as shown by the standard deviations of the sample replicates.

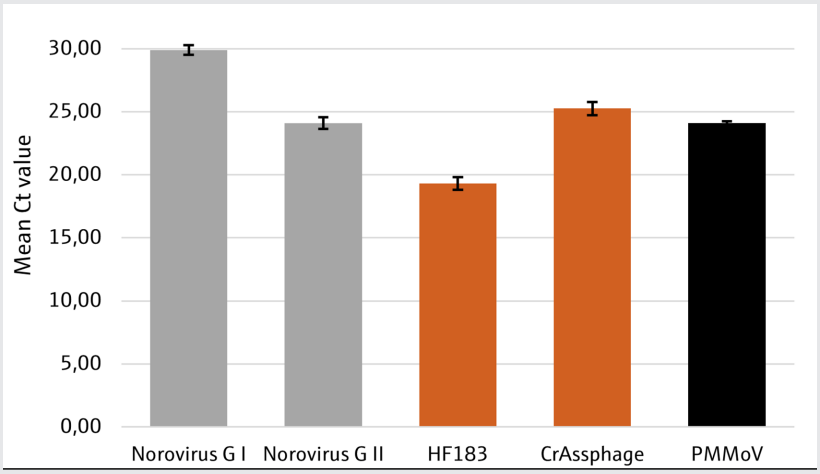

Figure 14: Overview of results of the three real-time PCR assays. Depicted are mean Ct values of five sample aliquots, each measured in duplicate, Error bars represent the standard deviation between sample replicates.

Conclusion

This application note demonstrates the successful adaption of the well-established workflow for the detection of SARS-CoV-2 in wastewater for novel target sequences. In addition, it shows that the process works both with commercially available PCR kits and with own developments. The improved workflow now includes a new sample transport medium further increasing analytical reliability while remarkably simplify handling: after concentration, material on the filter can be preserved and transported at room temperature and easily be released from the filter using the SpeedMill PLUS.

All scrutinized target sequences of norovirus, CrAssphage, HF183, and PMMoV were reliably detected in the tested wastewater samples. The analytical process is highly reproducible and shows very low standard deviations within replicates due to the excellent performance of the automated extraction platform InnuPure C16 *touch* and the real-time PCR cycler qTOWER^3. These results underline the suitability of the Analytik Jena's analytical equipment for nucleic acid-based epidemiology in the challenging matrix of wastewater.

Figure 15: InnuPure C16 *touch* and qTOWER^3

References

[1] Analytik Jena: Complete PCR-based Detection Workflow of SARS-CoV-2 in Wastewater, 2021

[2] W. Ahmed, S. Payyappat, M. Cassidy et al.: A duplex PCR assay for the simultaneous quantification of Bacteroides HF183 and crAssphage CPQ_056 marker genes in untreated sewage and stormwater; Environ Int. 2019 May; 126:252-259. doi: 10.1016/j.envint.2019.01.035

[3] P. D'Aoust, E. Mercier, D. Montpetit et al.: Quantitative analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19 incidence and prevalence; Water Res. 2021 Jan; 188:116560; doi: 10.1016/j.watres.2020.116560

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Version 1.0 · Author: AnKo
en · 03/2022
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