Challenge

Development of a complete analytical solution for wastewater monitoring with the application example SARS-CoV-2.

Solution

Functional and EU recommendation[3] compliant workflow from sampling to the analysis result for qPCR-based epidemiology in wastewater. Successful highly sensitive and reproducible detection of SARS-CoV-2 RNA in real samples.

Watch our video
„From waste to data"

www.analytik-jena.de/monitoring-movie

Complete Wastewater-based Epidemiology (WBE) Workflow for detection of SARS-CoV-2 via RT-qPCR

Introduction

Wastewater-based epidemiology has established itself as an effective tool in COVID-19 management: The detection of SARS-CoV-2 RNA in wastewater provides valuable information about the spread of the virus on different scales - from local hotspots up to entire catchment areas of water treatment plants. As a resource-saving method, the analysis of biological parameters in wastewater is generally a tried and tested means of monitoring public health without costly individual testing and can also be useful to anticipate new pandemics.[1] It is known that non-infectious SARS-CoV-2 RNA is excreted in the feces of infected individuals regardless of their clinical symptoms.[2] The sampling of wastewater can therefore provide information about the prevalence in the catchment area of the sewage treatment plant. The complex wastewater sample matrix places special demands on the nucleic acid extraction process, since DNA or RNA must be free of inhibitors for the subsequent pathogen detection via PCR. Analytik Jena has developed a functional process that takes on these challenges and can be used reproducibly and reliably for nucleic acid-based wastewater monitoring and provides a one stop shop for the devices used for sampling, extraction, and PCR. The process is proven in use and meets the requirements defined in the EU recommendation [3] for monitoring SARS-CoV-2 in wastewater. In addition, the components can be flexibly configured according to the needs of the analysis.

1. Sampling: In the sewage treatment plant, the Liquistation CSF48 sampler from Endress+Hauser takes a 24-hour mixed sample fully automatically. The E+H Liquiport CSP44 is available upstream as a mobile variant for local sampling. Endress+Hauser autosamplers guarantee representative sampling and their temperature-stabilized interim storage (at 4°C).

analytikjena
An Endress+Hauser Company

2. Concentration of the analysis material: Because the viral nucleic acids are highly diluted within the sample matrix, they must be enriched before the extraction process. This is done by filtration with subsequent release of the RNA from the filter material in the SpeedMill PLUS (Analytik Jena) using innuSPEED Lysis Tubes (IST Innuscreen).
3. Automatic nucleic acid extraction in the laboratory: Using the InnuPure C16 *touch* (Analytik Jena), nucleic acids, including the viral target RNA molecules, are extracted and any inhibiting substances are removed. Extraction quality is assured by the finely tuned extraction chemistry of the innuPREP AniPath DNA/RNA Kit (IST Innuscreen). At higher sample throughput, the CyBio FeliX eXtract from Analytik Jena (which can also automatically setup the PCR) can be used instead of the InnuPure C16 *touch*. This increases throughput and reduces manual processing time.
4. Detection: With the qTOWER³, Analytik Jena provides efficient and powerful instrumentation for the amplification and identification of target sequences via polymerase chain reaction (PCR). In this application note, detection is performed using the IDEXX Water SARS-CoV 2 RT-PCR detection assay.

At the end of the process chain with its mutually compatible components, there is a reliable statement about how widespread SARS-CoV-2 is in the region under consideration. The process has therefore already been installed at many customers, including the Bauhaus University Weimar, the EGLV (Emschergenossenschaft-Lippeverband) and the Chemisches Untersuchungsamt Emden GmbH. Installation is possible in a small space, requires only minimal laboratory equipment and, after a short briefing, can also be executed by employees without advanced laboratory assistant training.
In the following sections, the detailed experimental conditions are detailed and results are presented. Finally, the further application potential is explained and it is shown that the technology is neither limited to the sample matrix wastewater nor to the detection of the pathogen of the COVID-19 pandemic.

Materials and Methods

Samples, Reagents and Consumables

- 100 mL sub-samples of 24 h mixed wastewater samples (here: 3 representative samples, Sample 1-3)
- Electro-negative filters, mixed cellulose ester membrane, hydrophilic, 0. 45 µm, ⌀ 47 mm (HAWP04700, Merck Millipore)
- innuSPEED Lysis Tubes J (845-CS-1120100, IST Innuscreen)
- DNA/RNA Shield (R1100-250, Zymo Research)
- innuPREP AniPath DNA/RNA Kit - IPC16 (845-IPP-8016096 oder 845-PPP-8016096, IST Innuscreen)
- Water SARS-CoV-2 RT-PCR Test (99-0015314, IDEXX)
- AccuPlex SARS-CoV-2 Verification Panel (0505-0168, LGC seracare)

Instrumentation

- Liquistation CSF48 (71093061, Endress+Hauser)
- SpeedMill PLUS (845-00007-2, Analytik Jena)
- InnuPure C16 *touch* (845-00020-2, Analytik Jena)
- qTOWER³ touch incl. color module 1 (844-00555-2, Analytik Jena)
- Color module 2 for qTOWER³ (844-00521-0, Analytik Jena)
- Stainless steel pressure filter cartridge (16249, Sartorius)
- Compressor (DMP180Z, Makita)
- Centrifuge (for 2 mL tubes)
- Vortex mixer
- Pipets
- Tweezers

Methods

With the CSF48 Liquistation from Endress+Hauser, a mixed wastewater sample was collected over a period of 24 hours as individual samples of 50 mL every five minutes (total: 14.4 liters). Then, a partial sample (100 mL) was passed through an electronegative filter in a stainless-steel pressure filter cartridge at a pressure of approx. 8 bar. The virus particles or viral RNA adhere to the filter material and must then be transferred to a small-volume solution. Therefore, the filter was cut into two halves of roughly the same size and each half was folded into an innuSPEED Lysis Tube J after it had been folded three times. Two of the Lysis Tube Beads were placed on the bottom of the tube before inserting the filter, the rest placed on top of the filter pieces. Finally, 1 mL of DNA/RNA Shield was added to each Lysis Tube. When using the SpeedMill PLUS in continuous mode for two minutes, the beads in the Lysis Tubes pelleted the filter material to release virus particles into solution. The lysis tubes were then centrifuged at 10,000 rpm for two minutes. After transfer to fresh tubes, these were centrifuged again with the same settings to produce a clear supernatant. From each replicate tube, 200 µL of the clear supernatant were

combined (total: 400 µL) and used for the extraction of the viral RNA with the InnuPure C16 *touch*. The innuPREP AniPath DNA/RNA Kit - IPC16 is used for the extraction, as it is optimized for the extraction of bacterial and viral DNA and RNA from different starting materials. The kit instructions for protocol 2 ("Isolation from 400 µL cell-free body fluids") were followed without modification. The optional carrier mix included in the kit was not used, but the internal control included in the PCR test was added to the sample. The latter is used to validate the extraction and the real-time PCR reaction: in any case, it leads to a detectable amplification and thus proves the proper functionality of the analysis even in the absence of SARS-CoV-2 in the sample. The nucleic acids were eluted in 100 µL of RNase-free water. On the qTOWER^3, the samples were analyzed in duplicate for the presence of the SARS-CoV-2 target sequence using the Water SARS-CoV-2 RT-PCR test. The SARS-CoV-2 targets (N1 and N2) are detected using FAM-marked probes with color module 1 of the qTOWER^3. As an internal control, the human RNase P gene is detected with a HEX-labeled probe using color module 2 (HEX_2). A positive control (PC) and a no template control (NTC) were also included in the PCR setup to confirm the correct progression of the reaction and the absence of contamination in the reaction mixture. Figure 1 shows the PCR profile used for the detection reaction. Figure 2 shows the settings used for fluorescence detection of the amplification signals. Standards with a known number of copies (10^3, 104, 105/mL) were used for assay calibration. They were extracted in the InnuPure C16 *touch* and used in the qPCR reaction analogously to the filtered and concentrated wastewater samples. The measurement was carried out in triplicate and served as the basis for calculating a calibration line in the qPCRsoft software, which was then used to determine the concentrations of the wastewater samples.used.

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

	4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		50,0	15:00	--	---	--,-	---	8,0
	2		95,0	01:00	--	---	--,-	---	8,0
45x[	3		95,0	00:15	--	---	--,-	---	8,0
	4	◆	60,0	00:30	3	44	--,-	---	6,0
	5								
	6								
	7								
	8								
	9								
	10								

Figure 1: Temperature-time protocol of the Water SARS-CoV-2 RT-PCR test (IDEXX) on the qTOWER^3 (Analytik Jena) Step 1: reverse transcription, step 2: initial denaturation, steps 3 + 4: amplification (denaturation + elongation). Data acquisition (scan) occurs during step 4.

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	HEX_2	5	◆	
3	Yellow	TAMRA	5		
4	Orange	ROX	5		
5	Red	Cy5	5		
6	NIR 1	Cy5.5	5		

Meas. repeats: 3 Color compensation: Off

Figure 2: qTOWER^3 scan settings in qPCRsoft. The blue color channel (FAM) was used for the SARS-CoV-2 targets (N1/N2) and the green channel (HEX_2) for the signal detection of the internal control.

Results and Discussion

The representative wastewater samples were collected at the inlet of a sewage treatment plant. They were taken during a SARS-CoV-2 high incidence phase at the end of December 2021 and beginning of January 2022, when the local 7-day incidence averaged approx. 500/100,000 inhabitants. Technically, the detection method worked flawlessly: the positive control for the N gene detection, with a Ct value of approx. 30, corresponds exactly to the value expected by the kit manufacturer (Table 1 and Figure 3).

Table 1: Ct values of the N gene amplification curves of the SARS-CoV-2 target sequence. The controls (positive control and NTC) were measured in single determinations, the wastewater samples in duplicate. Mean Ct indicates the mean of the duplicate determination, Std.Dev. Ct its standard deviation.

Sample name	Sample type	Dye	Gene	Ct	Mean Ct	Std.Dev. Ct
No Template Control	NTC	FAM	N Gene	No Ct		
Positive Control	Positive control	FAM	N Gene	29,85	29,85	0
Wastwater Sample 1	Unknown	FAM	N Gene	27,23	27,29	0,09
Wastwater Sample 1	Unknown	FAM	N Gene	27,35	27,29	0,09
Wastwater Sample 2	Unknown	FAM	N Gene	28,18	28,16	0,02
Wastwater Sample 2	Unknown	FAM	N Gene	28,15	28,16	0,02
Wastwater Sample 3	Unknown	FAM	N Gene	29,03	29,02	0,01
Wastwater Sample 3	Unknown	FAM	N Gene	29,02	29,02	0,01

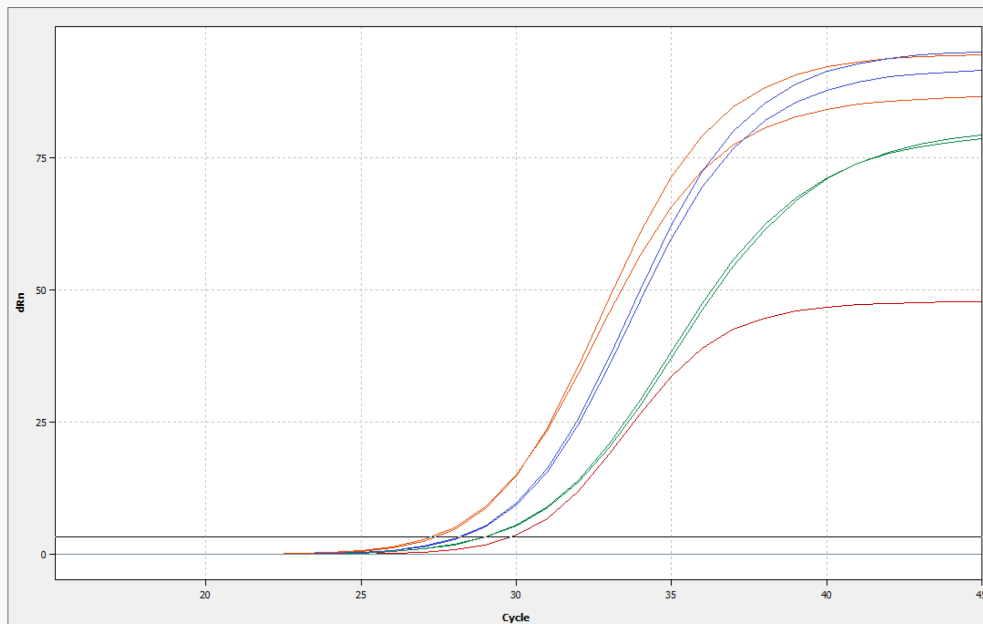

Figure 3: Amplification curves of the FAM-tagged SARS-CoV-2 target sequences (N gene). The negative control shows no amplification (zero level gray line) as expected, the amplification curve of the positive control (red curve) crosses the threshold (black horizontal line) at cycle 29.85. The values of the strongly positive wastewater samples (in duplicate: orange, blue and green curves) have threshold cycles between 27.23 and 29.03 and thus show a higher RNA concentration in the samples compared to the positive control used.

All samples are also positive in the internal control channel, demonstrating a successful extraction process (Table 2 and Figure 4). As expected, the NTC, which as a pure PCR negative control did not contain an internal standard, does not show a signal in any channel, which rules out contamination of the PCR mixture.

Table 2: Ct values of the amplification curves of the internal controls. The PCR controls (positive control and NTC) were measured in single determinations, the wastewater samples in duplicate. Mean Ct indicates the mean of the duplicate determination, Std.Dev. Ct its standard deviation.

Sample name	Sample type	Dye	Gene	Ct	Mean Ct	Std.Dev. Ct
No Template Control	NTC	HEX_2	IC	No Ct		
Positive Control	Positive control	HEX_2	IC	27,41	27,41	0
Wastwater Sample 1	Unknown	HEX_2	IC	26,81	26,79	0,03
Wastwater Sample 1	Unknown	HEX_2	IC	26,77	26,79	0,03
Wastwater Sample 2	Unknown	HEX_2	IC	27,98	28	0,02
Wastwater Sample 2	Unknown	HEX_2	IC	28,01	28	0,02
Wastwater Sample 3	Unknown	HEX_2	IC	29,24	29,29	0,06
Wastwater Sample 3	Unknown	HEX_2	IC	29,33	29,29	0,06

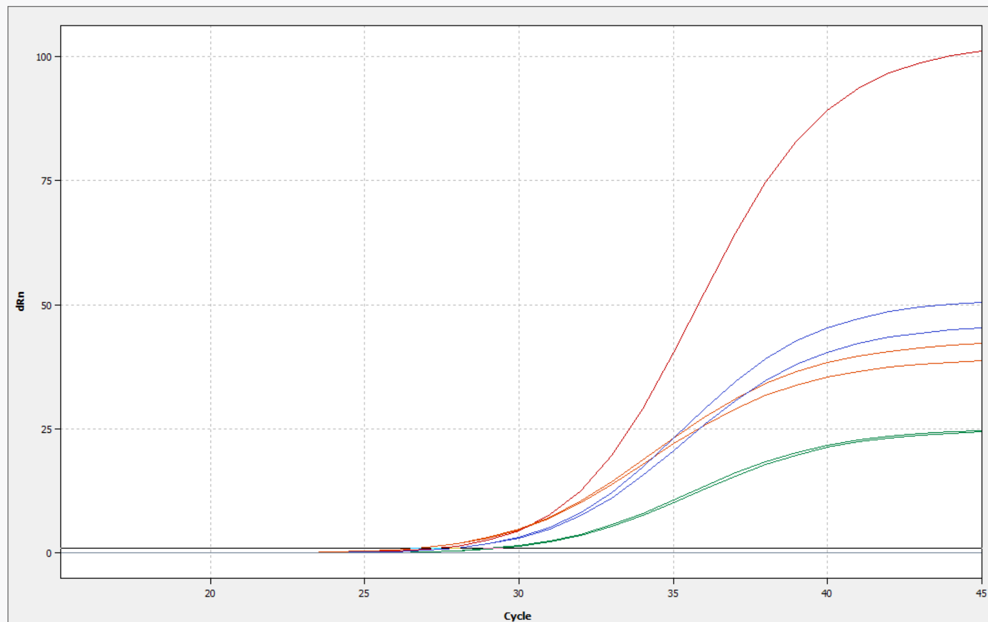

Figure 4: Amplification curves of the HEX-labeled internal control. The negative control shows no amplification (zero level gray line) as expected, the amplification curve of the positive control (red curve) crosses the threshold (black horizontal line) at cycle 27.41. The internal control values for the effluent samples (in duplicate: orange, blue and green curves) have threshold cycles between 26.77 and 29.33.

In each case, the tested wastewater samples were strongly positive, which reflects the epidemiological situation at the time the samples were taken. The determined Ct values correspond to the cycle in which the fluorescence signal rises above a defined threshold value. Since the PCR method doubles the amount of target nucleic acid and thus the fluorescence signal with each cycle, lower Ct values correlate with a higher initial amount of target molecules and thus a higher viral load in the sample.

Accordingly, in the example shown, wastewater sample 1 shows the highest viral load. The calibration associated with the run (not shown here) results in the following values for the viral load in the initial samples:

Sample 1:	Ct = 27.3	corresponds to	1.2 × 106 copies/L,
Sample 2:	Ct = 28.2	corresponds to	6.3 × 105 copies/L,
Sample 3:	Ct = 29.0	corresponds to	3.7 × 105 copies/L.

According to the explanation given above, and in line with the way PCR works, these values also illustrate how the viral load halves when the Ct increases by one unit. Based on the typical flow rate of a medium-sized sewage treatment plant (20,000 m^3), the determined values correspond to a total load of virus RNA in the order of several 1000 billion (1012) copies per day.

The process is not limited to periods when high levels of SARS-CoV-2 RNA are present in the wastewater. The qPCR technique works reproducibly up to Ct values of approx. 37 (depending on target, primers, and sample). It can also be used to detect amounts of virus that are more than 100 times lower. This makes the procedure suitable for pandemic monitoring even at low incidences – exactly at the level where political decisions to contain infection must be made. Figure 5 shows an example data set that was determined using the Analytik Jena wastewater workflow. The regional incidence resulting from the individual testing and the measured viral load show comparable courses over the sampling period. In particular, it has been shown that the test system signals a virus presence in wastewater in the range of the threshold values that are important for containment measures (e.g., 50) - and that with minimal measuring effort compared to individual testing.

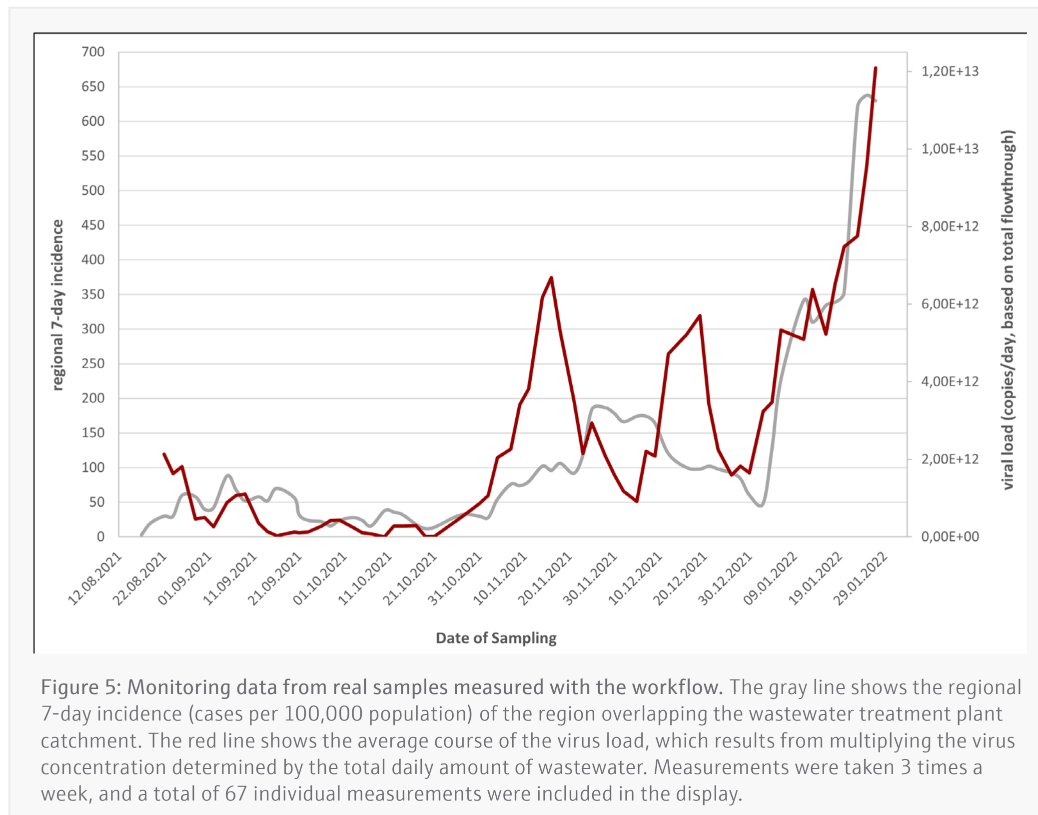

Figure 5: Monitoring data from real samples measured with the workflow. The gray line shows the regional 7-day incidence (cases per 100,000 population) of the region overlapping the wastewater treatment plant catchment. The red line shows the average course of the virus load, which results from multiplying the virus concentration determined by the total daily amount of wastewater. Measurements were taken 3 times a week, and a total of 67 individual measurements were included in the display.

Conclusion and Perspective

This application note describes the workflow for the extraction and detection of viral RNA from wastewater samples and explains how the data obtained can form the basis for epidemiological monitoring. The results presented show that the approach described here also ensures the detection of SARS-CoV-2 RNA from the demanding sample matrix wastewater. In addition, it could be demonstrated that its presence in the wastewater is representative of the prevalence in the collection area of the sewage treatment plant. Because the total of residents contributes to the result, the problem of underreporting associated with individual testing is not an issue with this monitoring approach. The automated and standardized sampling ensures the comparability of the samples. The cooling function of the CSF48 ensures the highest sample quality and integrity, which is of particular interest when working with viral particles and RNA. The automated extraction of RNA using the innuPREP AniPath DNA/RNA Kit - IPC16 with the InnuPure C16 *touch* provides reliably reproducible nucleic acid extracts. Finally, the qTOWER^3 enables fast and convenient virus detection using the Water SARS-CoV-2 RT-PCR test from IDEXX – software for data analysis included. In short, the process from a single source has proven itself in practice.
The quantitative real-time PCR used is considered the gold standard in molecular detection technology. Even more: the Analytik Jena workflow for the SARS-CoV-2 detection in wastewater with its devices, controls and quality standards fully meets the requirements for wastewater monitoring, which the European Union has defined regarding the performance of a workflow for systematic monitoring.[3] The most important aspects are stability, analytical sensitivity, and sufficient capacity for important process controls - the Analytik Jena workflow offers all of this.
Good to know: The method is not limited to detecting SARS-CoV-2 in wastewater! Other viruses such as norovirus can also be detected in wastewater.[4] In addition, it is possible to check water samples for human fecal contamination using known targets such as PMMoV, crAssphage or Bacteroides HF183.[4] The additional PCR detection reactions can be carried out starting from one and the same wastewater sample or its extracted nucleic acid – sometimes even simultaneously in the same PCR run (depending on the PCR reaction conditions). The process is also flexible regarding the sample matrix and can, for example, be designed for the detection of legionella in water from cooling circuits.[5]
In addition to the variety of possible applications, the workflow itself is highly flexible and can be scaled. At the sampling level, there is the option of using a mobile variant (Liquiport CSP44 from Endress+Hauser). With a higher number of samples, the CyBio FeliX eXtract from Analytik Jena can be used instead of the InnuPure C16 *touch*. This device can also automatically setup the PCR. This increases throughput and reduces manual processing time. The combination of reliable sampling with devices from Endress+Hauser, efficient extraction chemistry from IST Innuscreen and the high-performance equipment from Analytik Jena opens a wide range of possible applications in the field of Biosurveillance.

References:

[1] Sci Total Environ. 2020 Oc t 15;739:139076. doi: 10.1016/j.scitotenv.2020.139076. Epub 2020 Apr 30
[2] https://www.who.int/publications/i/item/WHO-2019-nCoV-IPC-WASH-2020.4
[3] https://ec.europa.eu/environment/pdf/water/recommendation_covid19_monitoring_wastewaters.pdf
[4] https://www.analytik-jena.de/fileadmin/content/upload/AppNote_qPCR_0008_en_wastewater_epidemiology.pdf
[5] https://www.analytik-jena.de/fileadmin/import/assets/12562424_AppNote_qPCR_0006_en_Legionella.pdf

Trademark Notice:
The brand names of the third-party products specified in the application protocol are usually registered trademarks of the respective companies or organizations.

Printing and further use permitted with reference to the source. © 2022 Analytik Jena GmbH
Publisher: Analytik Jena GmbH

This document is true and correct at the time of publication; the information within is subject to change. Other documents may supersede this document, including technical modifications and corrections.

Headquarters
Analytik Jena GmbH
Konrad-Zuse-Strasse 1
07745 Jena · Germany

Phone +49 3641 77 70
Fax +49 3641 77 9279

info@analytik-jena.com
www.analytik-jena.com

Version: 1.1 | Author: AnKo/ChGr/ChHe
en · 05/2022
© Analytik Jena GmbH | Pictures ©: Istock/tuachanwatthana