## Challenge

Highly sensitive detection of SARS-CoV-2 in representative wastewater samples.

## Solution

The Endress+Hauser group and Analytik Jena join forces to provide an easy to implement workflow starting with representative wastewater sampling, continuing with effective enrichment and nucleic acid extraction and closing with the powerful real-time PCR detection.

# Complete PCR-based Detection Workflow of SARS-CoV-2 in Wastewater

### Introduction

Biosurveillance of wastewater offers a time- and cost-effective tool to monitor public health in a comprehensive manner and can be useful to anticipate new pandemics[1]. Non-infectious SARS-CoV-2 particles are known to be shed with the feces of infected individuals, irrespective of their clinical symptoms[2]. Thus, sampling of wastewater can give insights into the prevalence within the gathering ground of the wastewater treatment plant.

However, with the sample material being very complex, the extraction and detection of the viral particles or other pathogens and microorganisms is far from trivial. In order to overcome the challenges, the following workflow can easily be implemented: For the collected samples to be representative, a 24 h composite sample is generated in a fully automated manner using the Liquistation CSF48 by Endress+Hauser. The highly diluted virus particles within the sample matrix need to be enriched prior to the extraction process. This is done with an electronegative filter (third party) from which the virus particles are subsequently freed utilizing innuSPEED Lysis Tubes in Analytik Jena's SpeedMill PLUS. The particle-free sample thus generated, is subsequently processed automatically using Analytik Jena's InnuPure C16 *touch*. In combination with the fine-tuned extraction chemistry of the innuPREP AniPath DNA/RNA Kit - IPC16 the efficient extraction of viral RNA (and/or DNA) and removal of inhibiting substances present in the sample are ensured. The workflow was successfully implemented with one of the wastewater treatment plants of the Emschergenossenschaft/Lippeverband. The RNA extracted on-site was used for the detection of viral target sequences. The amplification and identification of target sequences is reliant on fast and precise instrumentation in combination with a specific detection assay. Here, the Water SARS-CoV-2 RT-PCR Test by IDEXX was run on Analytik Jena's qTOWER$^3$ to yield optimal results.

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For providing representative samples the autosamplers from Endress+Hauser are the first component in a reliable wastewater biosurveillance setup. Using Analytik Jena's instrumentation for the whole workflow leading to the detection of viral particles in wastewater samples ensures the optimal compatibility between the single steps taken and generation of reliable results.

## Materials and Methods

### Samples, Reagents and Consumables

- innuSPEED Lysis Tubes J (845-CS-1120100, Analytik Jena)
- innuPREP AniPath DNA/RNA Kit - IPC16 (845-IPP-8016096 or 845-PPP-8016096, Analytik Jena)
- Water SARS-CoV-2 RT-PCR Test (98-0014718-00, IDEXX)
- Electronegative filters, mixed cellulose esters membrane, hydrophilic, 0.45 µm, Ø 50 mm (HAWP04700, Merck Millipore)
- 30% HCl
- Phosphate-buffered saline (PBS)
- PCR 96 well plate; white plastics
- 100 mL of a 24 h composite wastewater samples (total volume: 14.4 L); samples from 8 different 24-hour periods (labelled "A" through "G") were analyzed

### Instrumentation

- Liquistation CSF48 (71093061, Endress+Hauser)
- SpeedMill PLUS (845-00007-2, Analytik Jena)
- InnuPure C16 *touch* (845-00020-2, Analytik Jena)
- qTOWER$^3$ (e.g. 844-00555-2 (qTOWER$^3$ touch incl. color module 1); 844-00521-0 (color module 2), Analytik Jena)
- Stainless steel pressure filter holder (16249, Sartorius)
- Centrifuge (for 2 mL tubes)
- Vortex mixer
- Pipettes
- Forceps

### Methods

Various wastewater samples were collected over a period of 24 hours as composite samples of 50 mL every five minutes using the Liquistation CSF48 by Endress+Hauser. Over the 24-hour period the total volume of the sample is 14.4 L. The pH of 100 mL of this sample was adjusted to 3.5 – 4.0 using concentrated hydrochloric acid ($HCl_{aq}$). Subsequently, the 100 mL sample was passed through an electronegative filter in a stainless steel pressure filter holder using about 8 bar pressure. Viral particles adhere to the filter material and must subsequently be released into a small-volume-solution. This was accomplished by cutting the filter into six strips of roughly equal width and inserting the pieces into an innuSPEED Lysis Tube J. Two of the Lysis Tube beads were put at the bottom of the tube before inserting the filter, two further beads were placed on top of the filter pieces. Finally, 1 mL PBS was added to each Lysis Tube. Using the SpeedMill PLUS in continuous mode for two minutes the beads within the Lysis Tubes pelleted the filter material to release viral particles into solution.

Thereafter, the Lysis Tubes were centrifuged at 10.000 rpm for two minutes. After removing the beads and large filter particles, the tubes were centrifuged with the same settings once more to create a clear supernatant. 400 µL of the supernatant was used for the extraction of viral RNA with the InnuPure C16 *touch*. The innuPREP AniPath DNA/RNA Kit - IPC16 is used for the extraction procedure as it is optimized for the extraction of bacterial and viral DNA and RNA from various starting materials. The kit's instructions for protocol 2 (Isolation from 400 µL cell-free body fluids) were followed without modifications. Here, the Carrier Mix included with the kit was not used but can optionally be added to the samples (12.5 µL / sample). The nucleic acids were eluted in 100 µL RNase-free water. Using the Water SARS-CoV-2 RT-PCR Test on the qTOWER$^3$, samples were analyzed in duplicate for the presence of SARS-CoV-2 target sequence. The SARS-CoV-2 target (N1 and N2) is detected using a FAM-labelled probe with color module 1 in the qTOWER$^3$. As an internal control the gene for human RNase P is detected with a HEX-labelled probe using color module 2 of the qTOWER$^3$. The internal control is used to validate the extraction as well as the real-time PCR reaction. A Positive Control (PC) and a no-template control (NTC) were also included in the PCR setup to demonstrate the valid performance and absence of contaminations within the reagents 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 (by IDEXX) on the qTOWER³ (by Analytik Jena)
Step 1 – reverse transcription, step 2 – initial denaturation, steps 3 + 4 – amplification (denaturation + extension), data acquisition 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 | NIR1 | Cy5.5 | 5 | | |

Meas. repeats: 3 Color compensation: Off

Figure 2: Scan settings od the qTOWER³ in the qPCRsoft

## Results and Discussion

The extractions as well as the real-time PCR run were valid, as the controls yielded the necessary results: The Positive Control showed results for both targets, SARS-CoV-2 and the internal control. The NTC did not show any amplification for either target.

All the samples were valid as the internal control was detected with Ct values lower than 36 (see Figure 4 and Table 2), the limit given by the instructions of the detection assay. Samples A and F contained SARS-CoV-2 RNA above the limit of detection (see Figure 3 and Table 1). The results of Sample G are ambiguous as one of the duplicate results show a Ct value while the other has no Ct value. In all other samples both duplicates showed no amplification of the SARS-CoV-2 target sequence, indicating that SARS-CoV-2 RNA is absent from these samples or below the limit of detection.

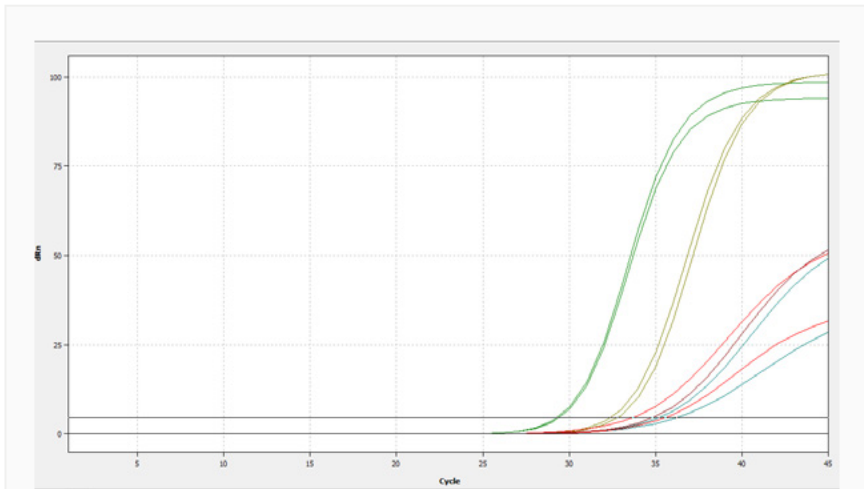


Figure 3: Amplification curves of the FAM-labelled SARS-CoV-2 target (N gene)
Two dilutions of the positive control (green: undiluted; ochre: 1:10 dilution) as well as various waste water samples were examined in duplicate. The corresponding Ct values are listed in Table 1.

Table 1: Ct values of SARS-CoV-2 target gene amplification curves

| Sample | Ct | |
|---|---|---|
| Sample A | 34.89 | 35.78 |
| Sample B | No Ct | No Ct |
| Sample C | No Ct | No Ct |
| Sample D | No Ct | No Ct |
| Sample E | No Ct | No Ct |
| Sample F | 33.27 | 35.17 |
| Sample G | 34,46 | No Ct |
| PC | 29,18 | 29,09 |
| PC 1:10 | 32.53 | 32.15 |
| NTC | No Ct | No Ct |

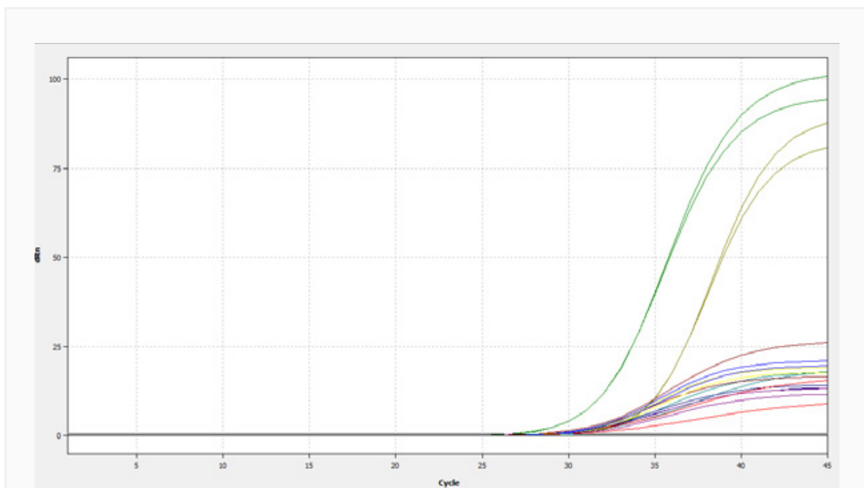


Figure 4: Amplification curves of the HEX-labelled internal control target
Two dilutions of the positive control (green: undiluted; ochre: 1:10 dilution) as well as various waste water samples were examined in duplicate. The corresponding Ct values are listed in Table 2.

Table 2: Ct values of Internal control amplification curves

| Sample | Ct | |
|---|---|---|
| Sample A | 29.46 | 29.37 |
| Sample B | 29.13 | 29.31 |
| Sample C | 30.19 | 29.46 |
| Sample D | 29.73 | 29.42 |
| Sample E | 29.37 | 29.23 |
| Sample F | 29.02 | 30.47 |
| Sample G | 28.57 | 29.02 |
| PC | 27.14 | 27.12 |
| PC 1:10 | 30.65 | 30.66 |
| NTC | No Ct | No Ct |

## Conclusion

This document details the workflow of the extraction and detection of viral RNA from wastewater samples performed by the Emschergenossenschaft/Lippeverband. The results obtained show, that the approach described here can ensure the detection of SARS-CoV-2 RNA even from challenging sample matrices. The detection within wastewater is representative of the spread of infection within the gathering ground of the wastewater treatment plant. The automated and standardized sample collection ensures the comparability of samples from day to day. The cooling function of the CSF48 ensures highest sample quality and integrity which is of special interest especially when working with viral particles and RNA. Furthermore, acidification prior to filtration may not necessarily be needed to yield good results as indicated by a recent study[3]. By scipping the acidification step the sample preparation may be further streamlined. While the automated extraction of the RNA using the innuPREP AniPath DNA/RNA Kit - IPC16 with the InnuPure C16 *touch* yields reliably reproducible yields. The qTOWER$^3$ enables the fast and comfortable detection of the target sequences within the eluted nucleic acids using the wastewater-specific SARS-CoV-2 RT-PCR Test by IDEXX.

By extension, the workflow described can also be applied for the extraction and detection of nucleic acids – DNA as well as RNA – of other pathogens. The combination of the reliable sample collection and versatile homogenization and extraction protocols with diverse detection assays open up a variety of possible applications within the field of biosurveillance. Furthermore, automated sample collection by the portable automatic water sampler Liquiport CSP44 (by Endress+Hauser) may be implemented into the here presented workflow, analougsly, in order to achieve flexible sampling of dedicated points of withdrawal as well as short-term sampling campaigns.

**References:**

[1] Sci Total Environ. 2020 Oct 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] Sci. Total Environ. 2020 Oct 15; 739:139960. doi: 10.1016/j.scitotenv.2020.139960.

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