Technical Guide Part 2 for Wastewater Surveillance – Molecular Biological Analysis

This Technical Guide is part of a set of four interrelated working documents that should be considered together:

- Technical Guide Part 1 for Wastewater Surveillance – Wastewater Sampling
- Technical Guide Part 2 for Wastewater Surveillance – Molecular Biological Analysis
- Technical Guide Part 3 for Wastewater Surveillance – Sample Logistics and Data Transmission
- Technical Guide Part 4 for Wastewater Surveillance – Data Processing

This guide describes the following areas:

1. **General Notes**
2. **Sample Treatment**
3. **Concentration and Extraction**
4. **Quantification of Viral Genome Copies using PCR**
5. **Quality Control of PCR Results**

1. General Notes

This guide provides the basis for the quantitative detection of specific gene fragments from various viruses, such as Severe Acute Respiratory Syndrome Virus Type 2 (SARS-CoV-2), Influenza A and B viruses, Respiratory Syncytial Virus (RSV) groups A and B, and universally detectable fecal viruses (fecal indicator viruses) through molecular biological methods, i.e., (reverse transcription) polymerase chain reaction ((RT-)PCR). It describes key control parameters essential for quality-assured analysis. It also lists information needed for later normalization of quantitative data, which should be continuously transmitted with the analyzed sample (monitoring data) to the state authority or the UBA database (see also Technical Guide Part 3 – Sample Logistics and Data Transmission).

The various steps of sample extraction and PCR analyses must be carried out by personnel trained in microbiology and molecular biology. Moreover, experimental phases (homogenization, sample concentration/virus enrichment, and nucleic acid extraction versus amplification and detection) must be spatially separated. Contamination should be avoided through the use of sterile, nuclease, and nucleic acid-free materials/reagents, with frequent disinfection and glove changes. Laboratories must optimize protocols to ensure quantification is possible even at low virus concentrations in wastewater samples. Recovery rates, as well as detection and quantification limits for each viral target gene, must also be determined.

2. Sample Treatment (Storage, Homogenization)

After sampling, wastewater should be processed as soon as possible. Temporary storage at 4°C is possible for a few days, though processing within 48 hours is recommended. Literature indicates gene fragment stability for up to 9 days in refrigerated samples (Markt et al., 2021). Significant concentration losses have been reported with (deep) frozen samples.

Nucleic acid extracts can be stored at 4°C if PCR analysis is performed shortly afterward. For longer storage (retention samples), -80°C is recommended. To avoid repeated thawing, aliquoting of samples is suggested.

In the lab, automated homogenization of samples in 1-liter transport containers is recommended by overhead shaking for 15 minutes. Only then should subsamples be taken for further analysis.

3. Concentration and Extraction

Virus particles or components can be concentrated through various methods. Table 1 summarizes the three most frequently applied methods within AMELAG.

A common initial step in many protocols is the separation of solid particles from raw wastewater by centrifugation (15-45 min, 4000-5000 x g), which facilitates the removal of solids, e.g. using filtration, polyethylenglycol precipitation or centrifugal ultrafiltration, and continues with the aqueous phase.

Table 1: Methods for Concentration of Viral Particles

Method	Starting volume	Options
Precipitation with Polyethylene Glycol (PEG)	40 mL - 1 L	Polyethylene glycol 8000 (PEG 8000) (10% w/v) (or PEG 6000) + NaCl (2.25% w/v)
Pressure or Vacuum Filtration	40 mL - 1 L	Negatively or positively charged filter membrane, pore size 0.1 µm to 0.45 µm
Centrifugal Ultrafiltration	40 mL - 200 ml	Various filter types and rotation speeds

Depending on method used and starting volume, limits of detection of targeted genes vary (see below). Important parameters for the choice of the appropriate concentration method are:

1) Maximal and minimal volumes which can be processed using the method
2) Efficacy
3) Suitability of the method for target virus
4) Available laboratory equipment
5) Usability and cost effectiveness

After concentration, viral or total nucleic acids should be extracted in high quality. Common extraction techniques include organic extraction with phenol, water, and alcohol, as well as silica-matrix binding, often available in kit form. Nucleic acids are typically eluted in 50-200 µL volumes. Purity can be assessed photometrically at wavelengths of 230, 260, and 280 nm or by additional methods like fluorescence measurements and fragment analyses. The efficiency of extraction can be verified by including a known quantity of inactivated virus particles of a different virus, such as bacteriophage MS2 or Murine Hepatitis Virus (MHV), as a process control.

Extracted nucleic acids can be stored at -20°C for approximately one month without concentration loss. For PCR analysis, freezing in aliquots is recommended to avoid repeated freeze-thaw cycles. Long-term storage should be at -80°C.

4. Quantification of Viral Genome Copies using PCR

Quantifying RNA viruses via RT-PCR requires the enzymatic conversion of RNA into complementary DNA (cDNA) using an RNA-dependent DNA polymerase, a process known as reverse transcription (RT). This step is often coupled with PCR in a "one-step" procedure. RT is challenging with environmental samples due to inhibitors (e.g., heavy metals, humic substances, insoluble salts) typically present in these samples, which are difficult to remove during nucleic acid extraction. Some cases benefit from a two-step RT-PCR, where cDNA synthesis and its amplification/quantification are separated, which can help mitigate inhibitor effects.

If quantification of cDNA does not happen immediately after RT, cDNA can be stored at -20 °C for a short time. To detect any issues with RT, an Inhibitor Control should be performed. Serial dilutions of nucleic acids can be performed to remove PCR-Inhibitors. Also, Nucleic acids can be treated with suitable techniques for inhibitor removal.

For virus detection, only specific genomic regions are amplified, rather than the complete genomes. The most common quantification methods are quantitative real-time PCR (qPCR), which provides relative quantification via an internal specific standard, and digital PCR (dPCR) or digital droplet PCR

(ddPCR), which offers absolute quantification through statistical evaluation of endpoint analyses. qPCR remains widely used due to established laboratory capability, though dPCR/ddPCR techniques are increasingly preferred for their high sample throughput and lower detection limits.

Positive and negative controls should be included in all PCR reactions to verify functionality and to avoid false-positive or false-negative results. All samples (including controls) must be analyzed at least in duplicate per qPCR plate. dPCR/ddPCR does not require technical replicates due to the high number of reactions within a single analysis. Viral gene concentrations are usually reported as "gene copies/L" or "genome copies/L."

4.1 SARS-CoV-2

SARS-CoV-2 is an enveloped RNA virus with a non-segmented, single-stranded RNA genome of approximately 30 kb.

Amplification targets include specific primers and probes also used in medical molecular detection of SARS-CoV-2. Target regions distinguish SARS-CoV-2 from other coronaviruses and include nucleocapsid (N1, N2, N3), envelope (E), RNA polymerase (RdRp), and open reading frame (ORF 1a/b) regions. Due to mutations in the spike (S) region, primers and probes for this area must be regularly validated.

For reliable detection, at least two suitable viral genomic regions must be identified.

4.2 Influenza A and B Viruses

Influenza A and B viruses are enveloped, single-stranded, negative-sense RNA viruses segmented into eight parts. These code for various proteins, including highly conserved matrix (M1 and M2) and non-structural (NS1 and NS2) proteins, suitable for differentiation between Influenza A and B.

Influenza viruses A and B can be detected in nucleic acid extracts also used for SARS-CoV-2 quantification. Primer/probe systems, following WHO and CDC recommendations, are suitable for this purpose, as are those commonly used in medical laboratories. For further differentiation, e.g. the targeted identification of avian influenza like H5N1, an additional essay should be used when necessary.

4.3 RSV A and B

Das humanpathogene Respiratorische Synzytial-Virus (RSV) ist ebenfalls ein behülltes Virus. Das Genom dieses Virus besteht aus einer nicht segmentierten, einzelsträngigen RNA negativer Polarität mit einer Länge von etwa 15,2 kb. Es kodiert für 11 Proteine: zwei Nichtstrukturproteine (NS1, NS2), ein Nukleoprotein (N) ein Phosphoprotein (P), ein Matrixprotein (M), drei transmembrane Oberflächenproteine (SH, G, F), zwei Transkriptions-relevante Proteine (M2-1, M2-2) und eine Polymerase (L).

RSV hat einen einzigen Serotyp, der in zwei Hauptuntergruppen unterteilt ist, RSV A und RSV B, die jeweils mehrere Genotypen umfassen und deren Zuordnung auf der hochvariablen C-terminalen Region des G-Gens (HVR2) beruht. RSV verschiedener Genotypen können während einer einzigen Epidemie innerhalb derselben Gemeinschaft zirkulieren.

In der Literatur finden sich Primer/Sonden-Systeme die auf den Nachweis von konservierten Genen wie Polymerase (L), Nukleoprotein (N) oder Matrixprotein (M) gerichtet sind. Einige Systeme ermöglichen nur eine gemeinsame Erfassung der zwei Hauptuntergruppen, während andere eine Differenzierung nach RSV A und RSV B erlauben.

Abwasserproben, die mit RSV A- und RSV B-Viruspartikeln gespikt wurden, zeigten bei Anwendung etablierter Extraktionsmethoden akzeptable Wiederfindungsraten. Im Vergleich zur wässrigen Phase konnten in Extrakten der entsprechenden partikulären Phasen der mit RSV A bzw. RSV B gespikten Abwasserproben nur sehr geringe Virus-Konzentrationen bestimmt werden, sodass die Eignung der routinemäßig für den SARS-CoV-2-Nachweis aus der Flüssigphase gewonnenen Extrakte auch für eine RSV A und RSV B Quantifizierung gegeben sein dürfte.

Weitere Validierungen sind noch nötig und in Bearbeitung, um ein eindeutiges Verfahren beschreiben zu können.

RSV (Respiratory Syncytial Virus) is an enveloped virus with a single-stranded, negative-sense RNA genome encoding 11 proteins on 15,2 kilobases: two non-structural proteins (NS1, NS2), one nucleoprotein (N), one phosphoprotein (P), one matrix protein (M), three transmembrane surface proteins (SH, G, F), two transcription proteins (M2-1, M2-2) and one polymerase (L). It comprises one serotype with two main subgroups (RSV A and B), each containing multiple genotypes. One single epidemic can readily contain more than one of those genotypes.

Detection is based on conserved genes, such as polymerase (L), nucleoprotein (N), or matrix protein (M). Wastewater samples spiked with RSV A and B demonstrated acceptable recovery rates using established extraction methods. RSV was recovered mainly in the aequous phase of wastewater samples. Only small amounts of RSV could be recovered from the solid phase. Thus, the commonly taken wastewater samples are suitable for RSV detection.

Additional validation to further assess the applicability of said methods is in progress.

5. Quality Control of PCR Results

A validated approach for quality control is to compare the viral genome copies (SARS-CoV-2, Influenza, RSV) with concentrations of other human fecal viruses, such as the intestinal bacteriophage CrAssphage or the plant virus Pepper Mild Mottle Virus (PMMoV). These control viruses, used in normalization, must be quantified with the same sample processing and detection methods (qPCR, dPCR, or ddPCR) as pathogenic viruses.

Quantified viruses for quality control can have additional use as normalization parameter.

!	During a series of measurements for a wastewater treatment plant, sampling, processing and method of detection shall not be changed. This keeps data comparable.

Contact information	Federal Environment Agency, abwassersurveillance@uba.de Robert Koch Institute, abwassersurveillance@rki.de

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