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Standard Operating Procedures for Wastewater Surveillance of Influenza viruses

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Influenza Surveillance in Wastewater:

Standard Operating Procedure for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

Contents:

Standard Operating Procedure (SOP) for

I. **Detection and quantification of Multiplex Influenza, RSV-A/B, and SARS-CoV-2 [RespiRFC kit] using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay developed by GenePath Dx for wastewater monitoring (Research use only)**

II. **Detection and quantification of Influenza A virus (IAV) and Influenza B Virus (IBV) in wastewater isolated RNA samples using multiplex reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay**

Leveraging RT-qPCR for Influenza Wastewater Surveillance

- *Early Outbreak Detection:* RT-qPCR allows rapid detection of influenza RNA in wastewater, providing early warning of outbreaks before clinical cases are reported.

- *Real-Time Monitoring:* The assay enables continuous monitoring of influenza levels in wastewater, facilitating real-time tracking of virus circulation and dynamics.

- *Variant Detection*: RT-qPCR can detect specific influenza variants, aiding in the identification of emerging strains and informing vaccine development and deployment strategies.

- *Complementing Clinical Surveillance:* RT-qPCR complements clinical surveillance by capturing asymptomatic and pre-symptomatic cases, providing a more comprehensive understanding of influenza prevalence in communities.

- *Resource Allocation: Data* from RT-qPCR assays guide resource allocation by identifying areas with increased influenza activity, allowing for targeted interventions and resource allocation such as vaccination campaigns and antiviral distribution.

- *Early Warning System:* The assay serves as an early warning system for seasonal peaks of influenza activity, enabling healthcare systems to prepare for increased demands and implement timely interventions.

In essence, RT-qPCR assays in influenza wastewater surveillance offer a precise and efficient means of early detection, continuous monitoring, and informed response to influenza outbreaks.

Standard Operating Procedure (SOP)

I. Detection and quantification of Multiplex Influenza, RSV-A/B, and SARS-CoV-2 [RespiRFC kit] using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay developed by GenePath Dx for wastewater monitoring (Research use only)

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1. Objective

To detect and quantitate Multiplex Influenza (Influenza A + Influenza B), Respiratory Syncytial Virus (A/B) - RSV-A/B, and SARS-CoV-2 in wastewater isolated RNA samples using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay developed by GenePath Dx for wastewater monitoring.

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2. General Precautions

1. This custom assay was developed by GenePath Dx for detection of Respiratory Syncytial Virus (RSV), Influenza and SARS-CoV-2 nucleic acids from human respiratory tract samples.
2. Reagents with different lot numbers should not be mixed.
3. This kit is intended for use by experienced clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures (preferably with infectious agents).
4. Lab-coat and powder free nitrile single-use gloves should be used at all stages of the analytical procedure. Contact with skin should be avoided throughout the procedure.
5. Before setting up the assay, work surfaces, pipettes, and tube stands should be cleaned with 70% ethyl alcohol. 1% hypochlorite or H_2O_2 may also be used for cleaning, but care must be taken to wipe away all traces of them prior to use. Hands should be washed after the processes.
6. Samples, controls, and tube used in the test should be regarded as infectious waste and be eliminated in accordance with national regulations on the disposal of infectious waste. Before discarding, all reagents, residual samples and other relevant materials should be sorted to different waste types (i.e., medical waste, industrial waste etc.) in compliance with the above-mentioned regulations.
7. Kit components should be stored at -20°C in the dark.
8. Do not freeze-thaw kit components more than 3 times. This may affect the sensitivity of the assay. If more than 3 freeze-thaw cycles are expected, we recommend aliquoting the reagents into smaller volumes.
9. Do not use kits that are beyond the expiry date printed on the kit box.
10. Before opening any tube, briefly centrifuge the tube to collect the solution at the bottom of the vial.
11. Ensure unidirectional workflow in the laboratory to prevent contamination by PCR amplicons.
12. Use a dedicated space and calibrated pipettes for setting up the assay. We recommend the use of filter barrier tips for all assay steps.
13. Master Mix should never be vortexed but should be mixed gently before use.

3. Introduction

COVID-19 is the coronavirus pandemic caused by a novel coronavirus strain, SARS-CoV-2, that emerged in the Wuhan region of China in late 2019. The disease is spread both by infected patients as well as by asymptomatic carriers, hence there is a need for a highly sensitive and specific diagnostic test that can detect the virus from patients and carriers.Like SARS-CoV-2, the Influenza virus is also contagious and causes respiratory illnesses showing similar or overlapping symptoms. It causes mild to severe illness, sometimes even leading to death. The three main types of Influenza viruses are Influenza A (Flu A), Influenza B (Flu B) and Influenza C. While Influenza-C causes mild illness and is not known to cause epidemics, Influenza A and Influenza B are responsible for major illnesses. It is therefore very important to have a diagnostic test which can detect either or both these infections. The Multiplex Influenza (Influenza A + Influenza B), RSV-A/B, and SARS-CoV-2 RT-qPCR kit determines the presence of SARS-CoV-2, Influenza (Influenza A + Influenza B), and RSV-A/B nucleic acids from respiratory tract samples in a quantitative manner. Our test will detect all subtypes of Influenza A and all strains of Influenza B. This includes seasonal and pandemic H1N1 and H3N2 and the Yamagata and Victoria strains of Influenza B. The test, however, does not distinguish between Influenza A and Influenza B and/or its subtypes. The Respiratory Syncytial Virus causes infections in the lungs and respiratory tract. RSV can cause severe infections in infants, premature babies, elderly people and immunocompromised individuals having a weak immune system. If the RSV infection spreads to the lower respiratory tract, the patient may suffer from pneumonia or bronchiolitis. This test can detect RSV-A and RSV-B however it cannot distinguish them.

4. Principle of the assay

- This kit is a 5-plex single-tube hydrolysis-probe-based (5' exonuclease reaction) real-time reverse transcription polymerase chain reaction (RT-qPCR) test capable of detecting the presence of RSV, SARS-CoV-2, Flu A and Flu B in clinical specimens.
- The test targets the RSV-A and RSV- B matrix (M) genes, Flu A matrix (M) gene, the Flu B non-structural (NS) gene and two independent SARS-CoV-2 specific genes (RdRP and N-gene) to increase confidence in diagnosis.
- The test also includes a primer-probe set targeting the human housekeeping gene 'RNaseP'. The RNaseP assay serves as an internal control that monitors the adequacy of sample collection, RNA extraction, and provides an indication of whether PCR-inhibitory substances may be present in the sample.
- The probe-primer sets used in this assay are proprietary modifications of assays reported in the literature that have been developed after extensive literature reviews, bioinformatic analysis.

5. Kit contents

(-10°C/-30°C)
RespiRFC-Standard 1 (5000 copies/µL)
RespiRFC-Standard 2 (500 copies/µL)
RespiRFC-Standard 3 (50 copies/µL)
Master Mix
RespiRFC Probe-Primer Mix
Non-template Control - Nuclease Free Water

6. Materials required

1. Powder free nitrile gloves.
2. Calibrated pipettes (for dispensing volumes of 0.2-2 µL, 1-10 µL, 2-20 µL, 20-200 µL and 100-1000 µL).
3. Filter barrier tips.
4. 1.5 - 2mL tube stand.
5. Vortex mixer.
6. Cool racks to hold PCR tubes and Microcentrifuge tubes.
7. 1.5 ml/ 2ml micro centrifuge (MC) tubes
8. 0.2 ml PCR grade tubes with ultra-clear attached cap/ 0.2 ml PCR grade 96 well plates.
9. qPCR machine (***QuantStudio™ 5 Real-Time PCR System)***
10. 70% ethyl alcohol, and distilled water to clean work surfaces.
11. 1% bleach solution for waste disposal.

7. Assay Procedure

7.1 Pre-PCR steps:

1. Ensure that the cooling block is between 2-8°C.
2. Thaw all reagents before use and mix well by pulse-vortexing the tubes and briefly centrifuging them. DO NOT vortex the Master Mix. Gently tap-mix the tube and centrifuge briefly prior to use.

7.2 Preparation of Run Time Mix:

1. If 'n' is the number of clinical samples being tested, then prepare sufficient run time mix for 'n+4 reaction'. The four extra reactions are for the three standards and the NTC. Always prepare at least 5% excess run time mix to compensate for pipetting errors.
2. Prepare the PCR run time mix as outlined below:

Amount of Master Mix (µL)	Amount of Probe-Primer Mix (µL)	Total Volume (µL)
9.25	0.75	10.0

3. The required amount of Master Mix and Probe-Primer Mix must be calculated by user according to ratio given above. Positive and Non-template Controls and 5% overage must be considered.
4. The total reaction volume per tube is **15 µL** (10 µL prepared run time mix + 5 µL of sample).
5. Aliquot 10 µL of the Run Time mix into the PCR tube/strip/plate.
6. Add 5 µL of the sample into the Run Time Mix and gently pipette mix the contents.
7. Close the caps of the PCR tubes/strip/seal the PCR plate. Spin down the tubes/strip/plate and place them in the thermal cycler. Set the thermal cycling conditions per cycling condition table below.
8. Note: After preparing the run time mix, keep all the enzymes and reagents back in the freezer at - 10°C to - 20°C. The reagents of the kit have been tested with 3 freeze thaw cycles. If you wish to use the reagents with smaller batches of samples, consider aliquoting the reagents into multiple tubes on receipt.

7.3 Setting up the PCR:

Note: Set the reaction volume to **15 µL** while setting up the PCR conditions on the PCR machine.

7.3.1 RT-qPCR Cycling Conditions

Step	Stage	Temperature	Time
Pre-incubation	Hold	30°C/37°C	5 min
Reverse Transcription	Hold	52°C	7 min
RT Inactivation	Hold	95°C	3 min 30 sec
PCR	Cycling (42 cycles)	95°C	5 sec
		58°C	35 sec (Acquire fluorescence at all channels indicated in the next table)

7.3.2 Required Settings for Real Time PCR Instruments

Target	**Channel (Dye)**	**Fluorophore Excitation wavelength (nm)**	**Fluorophore Emission wavelength (nm)**
RSV-A + RSV-B	FAM	~495	~520
SARS-CoV-2 (RdRP + N)	HEX/VIC	~535	~560
Influenza A + Influenza B	CalRed610/ROX	~590	~610
Human Control	Cy5	~645	~670

Note: For **Thermo/ABI systems**, **DO NOT USE** the auto threshold settings for analysis. Please set manual thresholds for all the channels. The passive reference dye should be set as "None".

8. Analysis and Reporting:

Analysis of data should be carried out only by individuals experienced with the technique and with real-time PCR data interpretation. Please follow the instrument manufacturer's recommendations for run and analysis settings. Below are the instructions for some specific instruments:

Thermo-ABI real-time PCR systems require manual adjustment of thresholds and Y-axes for all fluorophores individually. It is suggested that thresholds be adjusted so that they fall within the early parts of the exponential phases of the real-time amplification curves and above any background signal. The passive reference dye should be set as "None".

9. Validation of the assay:

<table>
<tr><th>Tubes</th><th>SARS-CoV-2 (Covid-19) (Yellow)</th><th>Influenza (Influenza A + Influenza B) (Orange)</th><th>RSV-A + RSV-B (Green)</th><th>Human Control (Red)</th><th>Interpretation</th></tr>
<tr><td rowspan="2">Non-template Control</td><td>No amplification</td><td>No amplification</td><td>No amplification</td><td>No amplification</td><td>Run valid (no contamination)</td></tr>
<tr><td colspan="4">Amplification (one or more tubes)</td><td>Contaminated samples / workspace. Carry out a thorough decontamination and repeat with a freshly prepared reaction set.</td></tr>
<tr><td rowspan="2">Standards</td><td colspan="4">The number of calculated copies/ µL should fall in the range of ± 10% of the expected copies/ µL of each standard*</td><td>Run valid (Three Ct difference between standard 1, 2 and 3 should be maintained)</td></tr>
<tr><td colspan="4">No amplification (one or more tubes)</td><td>Run invalid. Repeat the entire assay</td></tr>
</table>

***Note:** When the logarithm of the concentration (Y-axis) is plotted against the Cycle threshold (Ct) value, the recommended range for the slope of the curve fit for the standards is between -0.25 to -0.35 and recommended regression value (R^2) > 0.98 for both the targets.

While calculating copies for an unknown sample manually, multiply the copies for the sample by 2.

Standards	Expected copies/ µL
RespiRFC Standard 1	5000
RespiRFC Standard 2	500
RespiRFC Standard 3	50

10. Interpretation of the results

Targets				Interpretation
SARS-CoV-2 (Covid-19)	**Influenza (Influenza A + Influenza B)**	**RSV-A + RSV-B**	**Human Control**	
No amplification	No amplification	No amplification	No amplification	*Invalid test:* Insufficient sample or the sample contains PCR inhibitors. Repeat testing is recommended. If results on repeat PCR are still showing invalid, then fresh sample collection is required. Please note, dilution of template (1:10) may mitigate inhibitors.
No amplification	No amplification	No amplification	Amplification (Ct ≤ 35)	*Negative for SARS-CoV-2, Influenza (Influenza A + Influenza B) and RSV-A + RSV-B*
Amplification (Ct ≤ 35)	No amplification	No amplification	Any result	*Positive for SARS-CoV-2 (Covid-19)*
No amplification	Amplification (Ct ≤ 35)	No amplification	Any result	*Positive for Influenza (either Influenza A or Influenza B)*
No amplification	No amplification	Amplification (Ct ≤ 35)	Any result	*Positive for RSV-A/B*
	No amplification			

Note:

a. Always check the quality of the amplification curve along with the Ct values before interpreting the result.
b. While co-infections are possible, they are relatively uncommon. Repeat testing from a fresh sample is suggested in cases where multiple targets are positive
c. For samples that are positive for Influenza A, reflex testing to determine the subtype may be considered.
d. This assay can detect human RSV-A and RSV-B but cannot differentiate between (as they are multiplexed in a single channel)

11. Limitations of the procedure:

1. The testing is limited to molecular genetics laboratories capable of performing and troubleshooting molecular diagnostics assays
2. Negative results do not rule out Covid-19/Influenza A/Influenza B infections in a sample. A false negative result may occur if a specimen is improperly collected, transported, or handled. False negative results may also occur if amplification inhibitors are present in the specimen, if the specimen has an inadequate pathogen load, or if the viral strain has acquired a mutation at the binding site of a primers/probe. In the typical course of a SARS-CoV-2 infection, there are two distinct 'window' periods – a short period at the start of the infection and a longer period at the latter stages of the infection – where the SARS-CoV-2 viral load may be below the threshold of detection. Therefore, repeat testing after 48- 72 hours may be considered in cases where suggestive clinical symptoms are persistent or there is a strong suspicion of exposure. Concomitant antibody testing can also help to differentiate whether the infection is in an early or later stage. Alternatively, an orthogonal assay (like viral genome sequencing) might be considered. Clinical decisions should be based on the clinical symptoms and their progression combined with the laboratory testing report.
3. Positive results are indicative of an active SARS-CoV-2 / Influenza A / Influenza B / RSV-A/B infection; however, positive results do not rule out additional bacterial infections or co-infections with other viruses (e.g. coinfections with viruses like Dengue and Chikungunya that have overlapping clinical presentations have been reported in certain regions of the world).
4. The laboratory performing the assay should follow good laboratory practices, should have the capability to handle infectious samples, and should follow proper waste disposal policy of biological samples.
5. Influenza H1N1 is a subtype of Influenza A and will be detected as Influenza A in this test without being specifically called out as H1N1.
6. This assay can detect human RSV-A and RSV-B but cannot differentiate between (as they are multiplexed in a single channel)

12. Reference

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5. https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.htmL
6. https://www.who.int/publications/i/item/laboratory-biosafety-guidance-related-to-coronavirus-disease-(covid-19)
7. https://www.cdc.gov/flu
8. Shu B, et al. Multiplex Real-Time Reverse Transcription PCR for Influenza A Virus, Influenza B Virus, and Severe Acute Respiratory Syndrome Coronavirus 2. Emerging Infectious Diseases. Vol. 27, No. 7, July 2021 (www.cdc.gov/eid), https://doi.org/10.3201/eid2707.2104

Standard Operating Procedure (SOP)

II. Detection and quantification of Influenza A virus (IAV) and Influenza B Virus (IBV) in wastewater isolated RNA samples using multiplex reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay.

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1. Objective:

- To detect the Influenza A virus (IAV) and Influenza B Virus (IBV) in wastewater isolated RNA samples using multiplex reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay
- To prepare serial fold dilution of both Influenza A virus (IAV) and Influenza B Virus (IBV) target inserted plasmid DNA by calculating the number of copies/ µl using Qubit dsDNA assay and digital PCR.
- To generate the Influenza A virus (IAV) and Influenza B Virus (IBV) linear standard curve from the corresponding serial fold dilution for its absolute quantification in wastewater isolated RNA samples.

2. Requirements:

1. 1.5 ml/ 2ml micro centrifuge (MC) tubes
2. 0.2 ml PCR grade tubes with ultra-clear attached cap/ 0.2 ml PCR grade 96 well plates with ultra-clear sealer
3. Well-calibrated micropipettes
4. Sterile filter tips
5. Vortexer
6. Micro centrifuge
7. Mini spin (0.2 ml tube adopter)/ Centrifuge (0.2 ml 96 well plate adopter)
8. qPCR machine (***QuantStudio™ 5 Real-Time PCR System)***
9. Qubit 4.0 Fluorometer ***(Invitrogen, catalog no: Q33238)***
10. Qubit™ dsDNA HS Assay Kit ***(Invitrogen, catalog no: Q32854)***
11. Nuclease free water ***(Ambion, Catalog no: AM9937, Invitrogen)***
12. Luna Probe One-Step RT-qPCR 4X Mix with UDG ***(NEB, Catalog no: M3019L)***
13. IAV and IBV primers ***(Sigma-Aldrich)***
14. IAV and IBV probes ***(IDT)***
15. IAV and IBV target inserted Plasmid DNA**
16. Wastewater isolated RNA samples

(**) IAV and IBV target inserted Plasmid DNA details

Plasmid DNA containing IAV and IBV target insert which can be amplified and suitable with the above primer pairs and probe was procured from *Centre for Cellular And Molecular Platforms (C-CAMP)* and used for the assay.

3. IAV and IBV primers and probe details [1]

Name of the sequence	Sequence 5'-3'
IAV Forward Primer 1(FP1)	CAA GAC CAA TCY TGT CAC CTC TGA C
IAV Forward Primer 2(FP2)	CAA GAC CAA TYC TGT CAC CTY TGA C
IAV Reverse Primer 1(RP1)	GCA TTY TGG ACA AAV CGT CTA CG
IAV Reverse Primer 2(RP2)	GCA TTT TGG ATA AAG CGT CTA CG
IBV Forward Primer	TCC TCA AYT CAC TCT TCG AGC G
IBV Reverse Primer	CGG TGC TCT TGA CCA AAT TGG
IAV Probe	5'-/FAM/TGC AGT CCT /ZEN/ CGC TCA CTG GGC ACG/3IABkFQ/-3'
IBV Probe	5'-/YakYel/CCA ATT CGA/ZEN/ GCA GCT GAA ACT GCG GTG/3IABkFQ/-3'

4. Assay Procedure

4.1.Procedure Notes:

- Wipe the working bench with 70% Ethanol before starting the experiment.
- Wear gloves throughout the entire procedure.
- In case of contact between gloves and sample, change gloves or sterilize with 70 % ethanol immediately to avoid contamination.

4.2.To detect the Influenza A virus (IAV) and Influenza B Virus (IBV) in wastewater isolated RNA samples using multiplex reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

- RT-qPCR assay preparation and its cycle conditions for IAV and IBV based on *[Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB, Catalog no: M3019L)]* and *QuantStudio™ 5 Real-Time PCR System* are as follows,

4.2.1. Reaction Setup procedure [2]

- Thaw Luna Probe One-Step RT-qPCR 4X Mix with UDG at room temperature, then place on cold rack at 4°C or ice.
- After thawing completely, briefly mix all components by inversion, pipetting or gentle vortexing.

- Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare assay mix of all components except RNA template, according to the table below.

Component	**Volume per 15 µL reaction**	**Final Concentration**
Luna Probe One-Step RT-qPCR 4X Mix with UDG	3.75 µL	1X
Forward Primer of IAV	6+0.6+0.6 µL	0.4 µM

(FP1, FP2) and IBV (10 µM)		
Reverse Primer of IAV (RP1, RP2) and IBV (10 µM)	6+0.6+0.6 µL	0.4 µM
Probe of IAV and	3 +0.3 µL	0.2 µM

IBV (10 µM)		
Template RNA	5 µL	< 1 µg (total RNA)
Nuclease-free Water	2.05 µL	

- Mix thoroughly but gently by pipetting or vertexing. Collect liquid to the bottom of the tube by brief centrifugation
- Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.
- Add RNA template to qPCR tubes or plate along with no template and positive control target inserted plasmid DNA standard dilutions. Seal tubes with flat, optically transparent caps or seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.
- Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 1600 rpm).

4.2.2. Programming the Real-Time Instrument

For faster results, the "Fast" ramp speed mode can be used where available (e.g., Applied Biosystems StepOnePlus®, QuantStudio®, 7500 Fast instruments).

Use **FAM** in dye selection and **None** in quencher selection for IAV, **VIC** in dye selection and **None** in quencher selection for IBV for (*QuantStudio™ 5 Real-Time PCR System)* and other required parameters based on qPCR instrument chosen.

The passive reference dye should be set as "ROX".

4.2.3. Cycle conditions:

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Carryover Prevention	25°C	30 seconds	1
Reverse Transcription	55°C	10 minutes	1
Initial Denaturation	95°C	1 minute	1
Denaturation Annealing, detection & Extension	95°C **56°C***	10 seconds 1 minute	45

*Note**: The above conditions and annealing temperature are optimized for mentioned master mix, positive standards and wastewater isolated RNA samples with considerable possibilities of the presence of inhibitors.**

4.3. To prepare serial fold dilution of Influenza A virus (IAV) and Influenza B Virus (IBV) target insert plasmid DNA by calculating the number of copies/ µl using Qubit dsDNA and digital PCR.

- Plasmid DNA containing the target insert concentration was quantified using Qubit dsDNA assay using the manufacturer's protocol.
- Number of copies of IAV and IBV plasmid/ µl from the obtained concentration can be calculated using the below formula [3],

Number of copies (Molecules) = [X ng * 6.0221 x 10^{23} molecules/mole] / [(N * 660 g/mole) * 1 x 10^{9} ng/g]

Where: X = amount of amplicon (ng)
N = length of dsDNA plasmid containing the IAV and IBV target insert
660 g/mole = average mass of 1 bp dsDNA
Calculate separately for IAV and IBV

- Prepare the 10-fold serial dilution of pool of IAV and IBV plasmid DNA in nuclease free water starting with 10^6 copies /µl to 10^3 copies /µl based on the above calculation.
- Cross-verify the exact copies of the any of the lower dilutions like 10^4 copies and 10^3 copies with the Digital PCR and accordingly back calculate the number of copies in the original stock.
- Prepare the 10-fold serial dilution of pool of IAV and IBV plasmid DNA in nuclease free water starting with 10^8 copies /µl to 10 copies /µl with the Digital PCR assessed copies count.
- Perform the multiplex RT-qPCR assay for the serially diluted target inserted plasmid DNA – 10^8 copies /µl to 10 copies /µl along with testing RNA samples.

Note: Vortex nicely and pipette accurately between each serial dilution for obtaining efficient known concentration standards

5. Analysis:

5.1.Data Analysis notes:

- Inspect the amplification plot to ensure that the threshold was set within the PCR initial exponential phase and above any background signal.
- Also check whether the amplification plot is proper with respect to multicomponent plot, not just proceed based on Ct values alone.
- Samples with the Ct values above 35 should be considered as negative.

5.2.To generate the IAV and IBV linear standard curve from the corresponding serial fold dilution for its absolute quantification in wastewater isolated RNA samples.

- Based on the known number of copies/ µl of IAV and IBV target inserted plasmid DNA and its corresponding Ct values, generate the linear standard curve separately for IAV and IBV from the Quant Studio Design and analysis software.
- Check the efficiency of the PCR and linearity of the standard curve based on slope and R^2 value (Acceptance criteria: The average value of the slope of the standard curves should be in the range of - 3.1 ≤ slope ≤ - 3.6, corresponding to amplification efficiencies of 110% to 90% and R^2 value for the individual standard curve should be ≥0.98 [4,5]
- With the help of the linear standard curve equation, either obtain the interpolated IAV and IBV concentration values of tested RNA samples directly from the software or interpolate manually using the linear curve equation obtained from the software.
- Back calculate the original number of copies/ µl based on the total elution volume for the samples used for extraction. Further back calculate the copies per mL of wastewater based on initial volume used for sample processing.

Note:

- Requirement of Qubit dsDNA assay and Digital PCR only necessary during the initial stage to calculate the number of copies IAV and IBV target insert plasmid.

- Based on the Ct values or number of copies/ µl range of overall samples, choose three/ four consecutive dilutions of IAV and IBV plasmid which will maximum cover the interpolating range of most of the samples tested.
- As well as that copies should be well detected with acceptable coefficient of variation (CV).
- Do not include LOD in the standard curve, unless it is detected efficiently with less CV and in linear range.
- Detect the LOD and decide the Ct cut-off accordingly (Lowest concentration at which 95% of the positive samples are detected with minimum 20 replicates tested). In other words, within a group of replicates containing the target at concentrations at the LOD, no more than 5% failed reactions should occur. Low-copy PCRs are stochastically limited, and LODs of <3 copies per PCR are not possible [4,5]
- Use that selected three dilutions with corresponding number of copies/ µl as standards for every future experiment and interpolate the samples IAV and IBV concentration accordingly. Aliquot the three/ four selected number of copies/µl of IAV and IBV standards in appropriate volume in multiple properly labelled tubes and store at -20° C. Avoid freeze-thaw more than three times.

6. Reference:

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