RT-qPCR Detection of Process Controls (Murine noroviurs and crAssphage) from Wastewater using AB 7500 V.1

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US Food and Drug Administration

This method was developed at the FDA's Center for Food Safety and Applied Nutrition for GenomeTrakr's pandemic response project, monitoring SARS-CoV-2 variants in wastewater. Protocols developed for this project cover wastewater collection, concentration, RNA extraction, RT-qPCR detection, library prep, genome sequencing, quality control checks, and data submission to NCBI. This protocol describes the murine norovirus (MNV; extraction control) and crAssphage (human indicator) RT-qPCR assay developed for use on the AB 7500 platform using software version 2.0 or 2.3. The assay incorporates an internal amplification control (IC) to prevent the reporting of false negatives due to inhibition or failure of the RT-qPCR. This multiplexed detection assay was developed for the determination crAssphage extracted from wastewater, as an endogenous control, and MNV as an extraction control. The assay is designed to be used in conjunction with the SARS-CoV-2 RT-qPCR detection assay. Valid sample results for SARS-CoV-2 detection are contingent upon the detection of the MNV extraction control from the sample being tested.

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GenomeTrakr, wastewater, SARS-CoV-2, crAssphage, murine norovirus, process control, extraction control, endogenous control, RT-qPCR, AB 7500 Fast

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Equipment and Supplies:

1. 7500 Fast Real-Time PCR System (ThermoFisher 4351106)
2. MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (ThermoFisher 4346906), or MicroAmp Fast 8-Tube Strip, 0.1mL (ThermoFisher 4358293)
3. MicroAmp Optical Adhesive Film (ThermoFisher 4311971), or MicroAmp Optical 8-Cap Strips (ThermoFisher 4323032)
4. Platefuge (Fisher Scientific NC1823435), or Stripfuge (USA Scientific 2621-0016), or equivalent for either
5. 96-well cool block (USA Scientific 4051-0525), or equivalent
6. Reagent cool block (USA Scientific 2312-2721), or equivalent
7. Adjustable calibrated micropipettes (0.2 – 1000 µl), two separate sets; one set dedicated for master mix setup and the other for template addition
8. Aerosol resistant pipet tips (0.2 – 1000 µl)
9. Personal microfuge (Labsource C90-044, or equivalent)
10. Hype-Wipe Disinfecting Towelettes (Fisher Scientific 14-412-56, or equivalent)
11. Vortex mixer (Labsource S16-200 or equivalent)

Reagents:

☒ Internal Control RNA **BioGX Catalog #750-0001** (Contact sales@biogx.com for ordering)

☒ Murine Norovirus **ATCC Catalog #VR 1937**

☒ One-Step RT-qPCR Kit **Qiagen Catalog #210210 or 210212**

☒ Nuclease-free water **Life Technologies Catalog #AM9937**

☒ Superase-In **Life Technologies Catalog #AM2696**

☒ FAM reference dye **Bio-rad Laboratories Catalog #1708780** , or equivalent

☒ Tris (1 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #AM9856**

☒ EDTA (0.5 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #AM9261**

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Primers and probe sequences in following table.

Custom Primers and Probes **IDT Technologies** .

Identification	Primers (Sequence 5' -> 3')	Location#
MNVR[a]	CACAGAGGCCAATTGGTAAA	6645-6626
MNVF[a]	TGCAAGCTCTACAACGAAGG	6520-6539
crAssF[b]	CAGAAGTACAAACTCCTAA	14363-14381
crAssR[b]	TGACCAATAAACAAGCCAT	14486-14468
IC46F[c]	GACATCGATATGGGTGCCG	N/A
IC194R[c]	AATATTCGCGAGACGATGCAG	N/A
Identification	**Probes**	**Location#**
MNVP	Cy5- AGACAAAAACCATTCAACGCCGGAGG -IB-RQ*	6578-6594
crAssPro	JOE- AATAACGATTTACGTGATGTAACTCGT -IB-RQ*	14404-14430
IACP[c]	TxRed- CCTTCCCGACCGATGGCATC -IB-RQ*	N/A

#based on accession nos. JF320650, MT006214.1, and NC_045512
[a]Hewitt, et al., 2009
[b]Modified from Stachler, et al., 2017
[c]Depaola, Jones, Woods et. al. 2010, U.S. Patent Application 0060166232
*IB RQ- Iowa Black RQ

Always wear gloves during this procedure and never wear the same gloves when going between master mix and samples.

Always use aerosol resistant pipette tips for PCR.

Assembly of master mix should be done in a designated Master Mix PCR hood or BSC that has been decontaminated with 10% Bleach solution or HypeWipes followed by 70% Ethanol, or similar product and UV irradiated for 20 minutes prior to use. RNA sample template should be added in a separate designated area, physically separated from the Master Mix hood/area. Equipment should not be shared between the two areas.

Master Mix Preparation

1 Prepare Master Mix for all sample and control reactions based on the **volumes per 25 µl reaction** in this table. Composition of mixes are listed here: Reagent Mixes for RT-qPCR

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Detection of Process Controls from Wastewater Samples (protocols.io) and should be prepared in advance and stored appropriately. Alternatively, Master Mixes can be prepared from individual components as described here: **Master Mix Table for MNV-crAss Assay.pdf**.

Reagent	Volume per 25 µl reaction
Buffer Mix	15.55 µl
Primer Mix	2 µl
Probe Mix	1 µl
Enzyme Mix	1.25 µl
FAM[A]	2 µl
Internal Control (IC) RNA*	0.2 µl
RNA	3µl

[A] Use a 1:1000 dilution (made in Primer TE) of FAM reference dye in the N1-N2-IC triplex assay.

*Amount varies with concentration of IC RNA. The amount of IC RNA template needs to be adjusted based on the prepared stock concentration to report a Cycle threshold (Ct) of 20-25 when no inhibition is present in the reaction (i.e., the negative control reaction).

Do not add IC or sample RNA at this step!

1.1 Thaw Master Mix reagents in bench top cool block (chilled at **2-8 °C**) or **On ice** in master mix preparation hood.

Keep Enzyme chilled continually; these enzymes are in glycerol and do not need to be thawed.

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1.2 Vortex reagent tubes for **00:00:03 +/- 1 sec** at setting medium high to high (if vortex has settings). 3s

1.3 Briefly centrifuge all reagents **00:00:05 +/- 2 sec** in a personal microcentrifuge to bring liquid to the bottom of tube. 5s

1.4 Return all reagents to bench top cool block (chilled at **2-8 °C**) or **On ice** .

2 Proceed to hood/area or room where the template is added and thaw IC RNA and sample RNA in this hood/area.

RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. A negative (water) and positive PCR control should be added to each reaction set-up.

2.1 Briefly centrifuge IC RNA **00:00:05 +/- 2 sec** in a personal microcentrifuge to bring liquid to the bottom of tube. 5s

2.2 Add appropriate volume of IC RNA (**0.2 µL** per reaction) to Master Mix from Step 1.4 in cold block/on ice.

2.3 Vortex briefly and centrifuge **00:00:05 +/- 2 sec** in a personal microcentrifuge. 5s

Reaction Set-Up

3 Add **22 µL** of Master Mix to each designated reaction tube or sample wells.

4 Briefly centrifuge sample RNA **00:00:05 +/- 2 sec** in a personal microcentrifuge to bring liquid to the bottom of tube.

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5 Add **3 µL** of sample RNA template to each of three reaction tubes or wells.

6 ⚠

Ensure each plate or run has appropriate controls (positive and negative controls) included.

Positive control prepared as described here: Positive Control Material for RT-qPCR of SARS-CoV-2 and Process Controls (protocols.io).

7 Seal sample plate or strip tubes. Then, briefly spin **00:00:05 +/- 2 sec** .

8 Start run on Applied Biosystems 7500 Fast instrument.

Assay parameters were optimized using the AB 7500 software versions 2.0-2.3. If other instruments or software versions are used, additional optimization may be needed.

8.1 Use the following settings for the Experiment Properties:
"7500Fast (96 wells)"
"Quantitation Standard Curve"
"TaqMan Reagents"
"Standard (~2 hours to complete run)"

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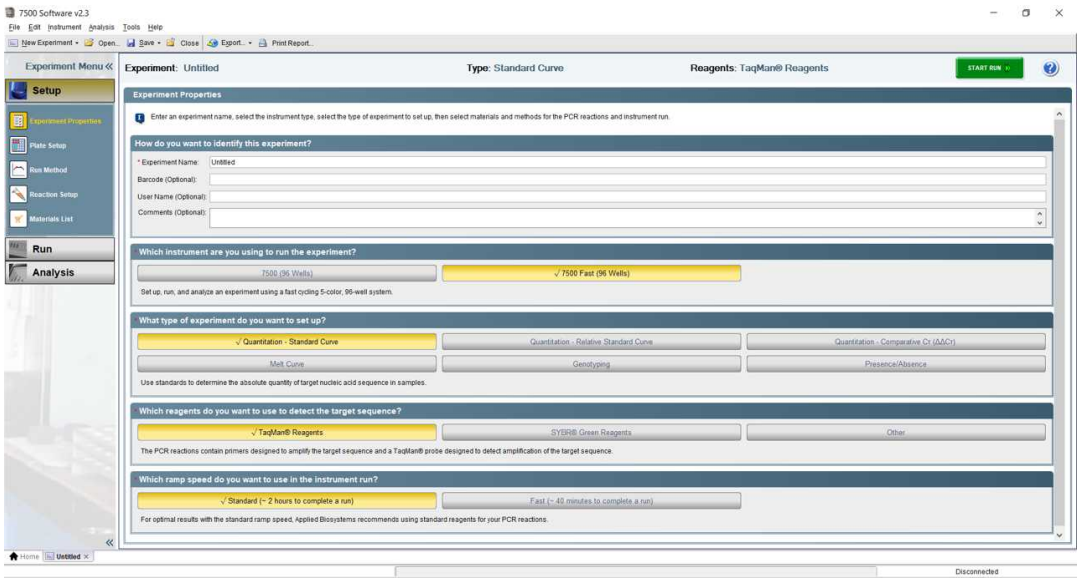

8.2 Identify the appropriate target reporters (MNV-Cy5, crAss-JOE, IC-TexasRed) and leave all quenchers as "NFQ-MGB".

8.3 Select appropriate passive reference dye (FAM for the triplex assay).

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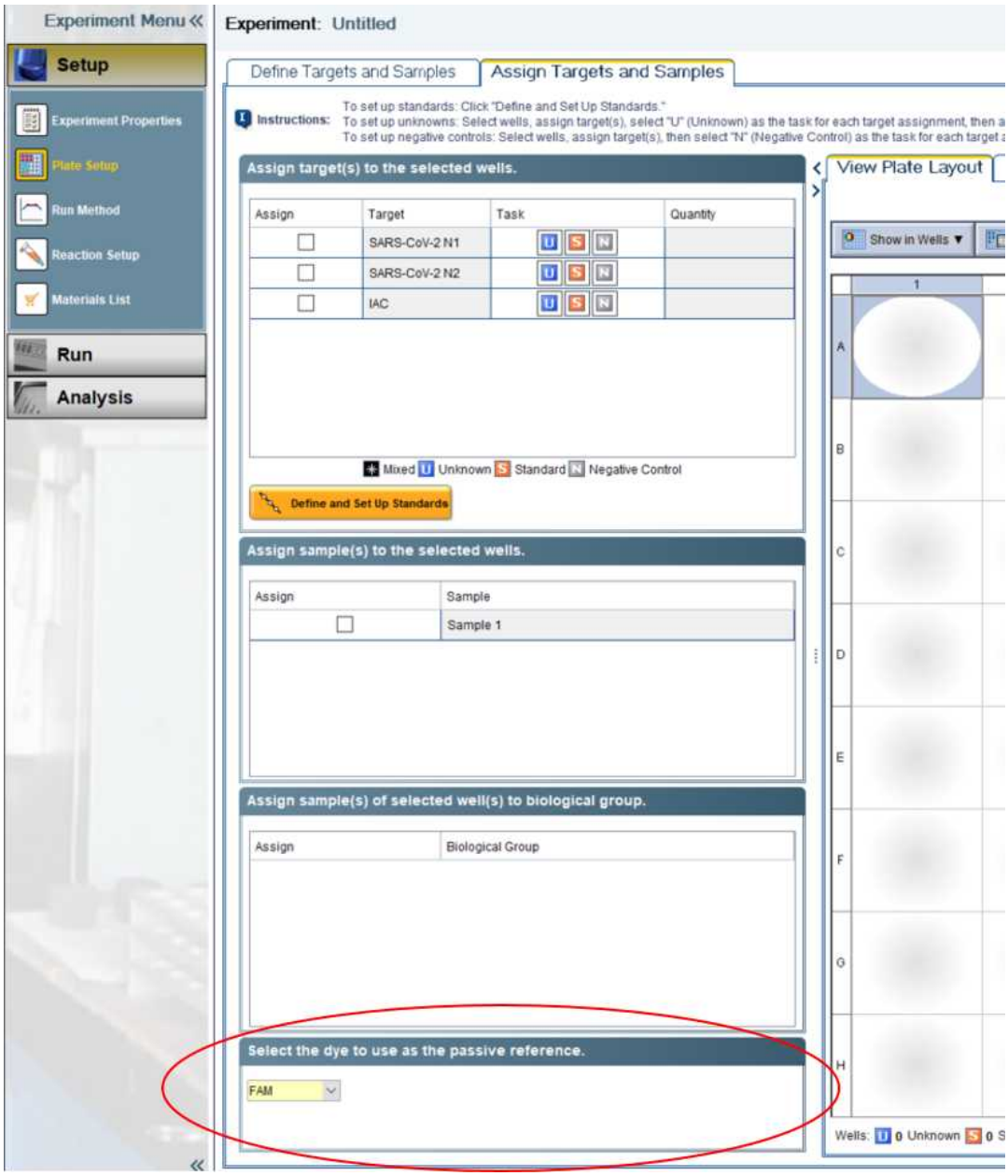
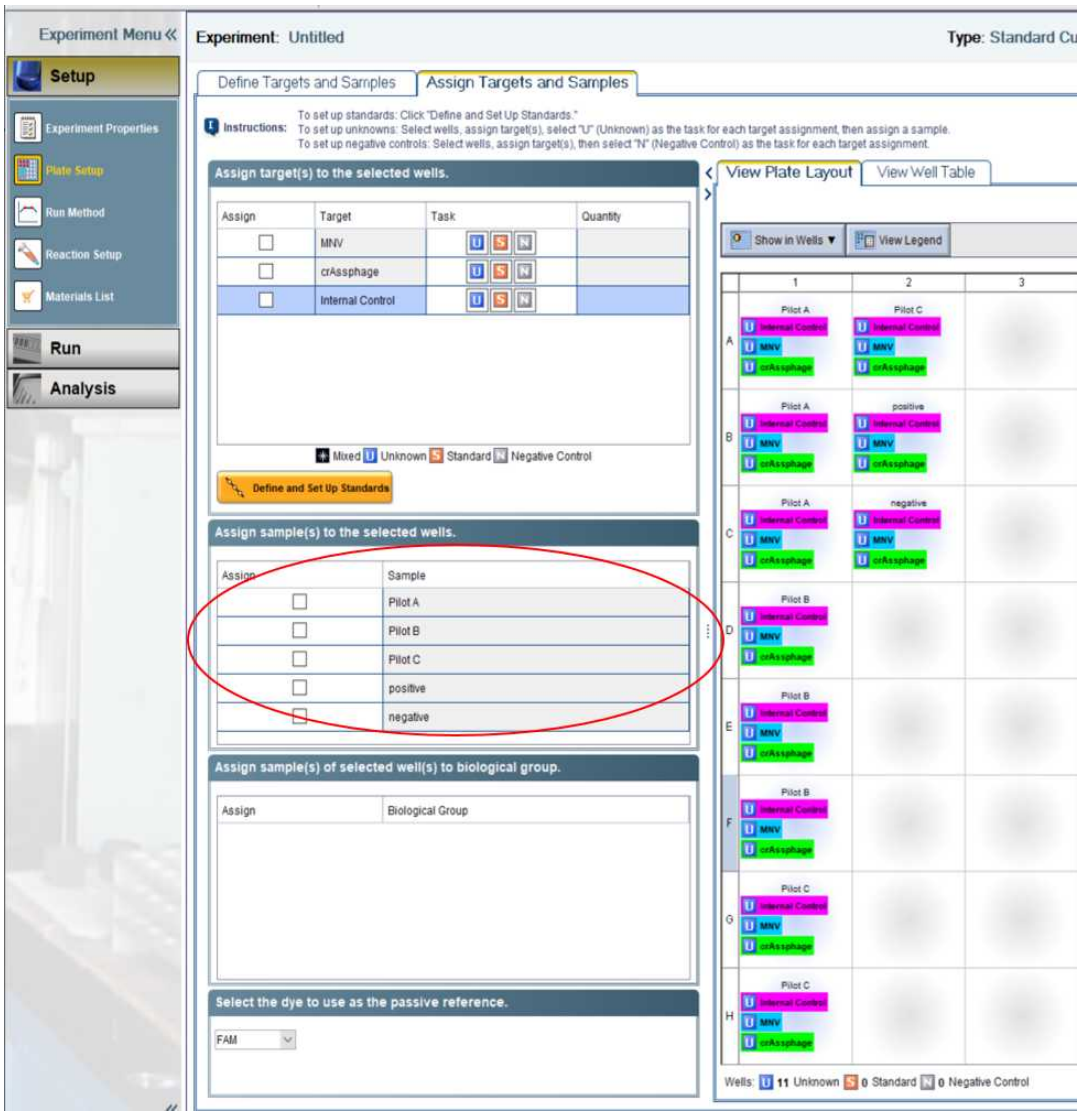


8.4 Assign targets and samples.



1h 6m 35s

8.5 Use the following settings for Run Method:

25 µL reaction volume

Holding stage 1: **50 °C** for **00:50:00**

Holding stage 2: **95 °C** for **00:15:00**

Cycling stage: 45 cycles of **95 °C** for **00:00:15**, **55 °C** for **00:00:20**, **62 °C** for **00:01:00**

Enable data collection on Step 3 of Cycling stage

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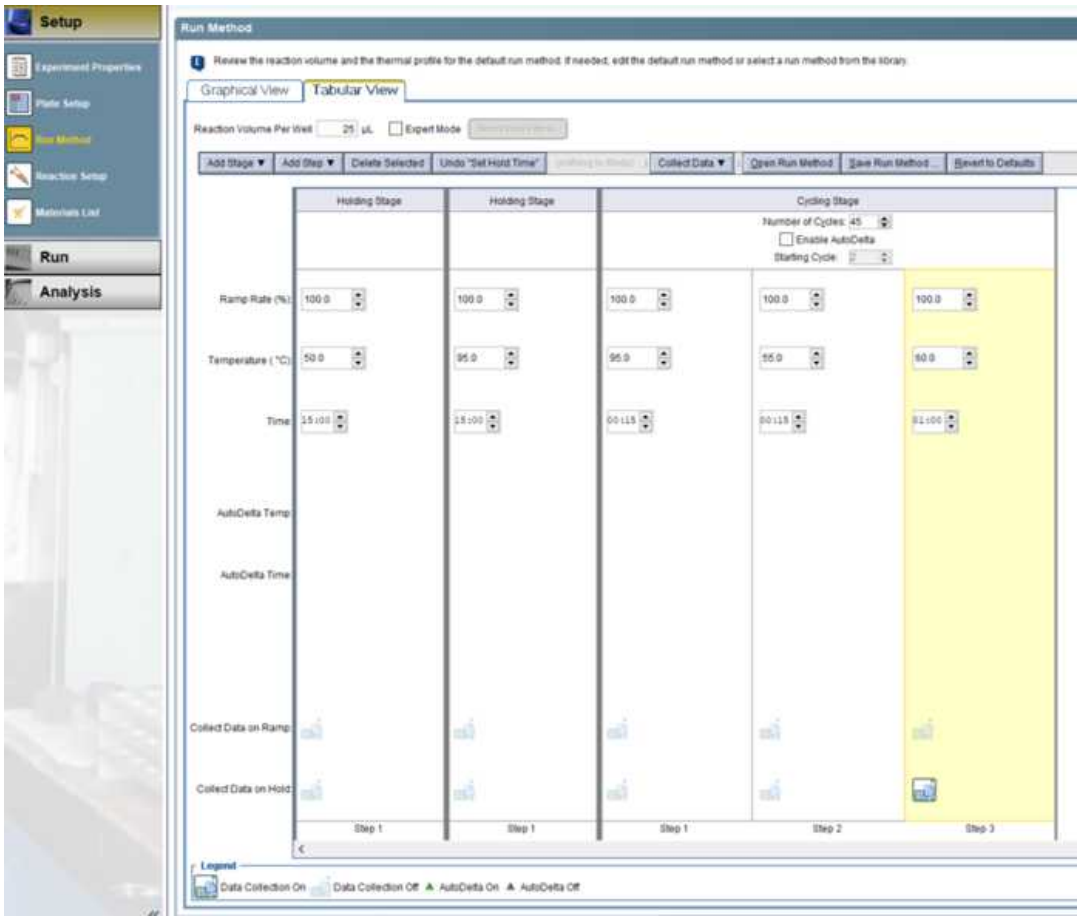

Data Analysis

9 Adjust analysis settings to appropriate thresholds. All thresholds should be set at **0.01**. Baseline start cycle should be set at 3 and baseline end cycle should be set at 10.

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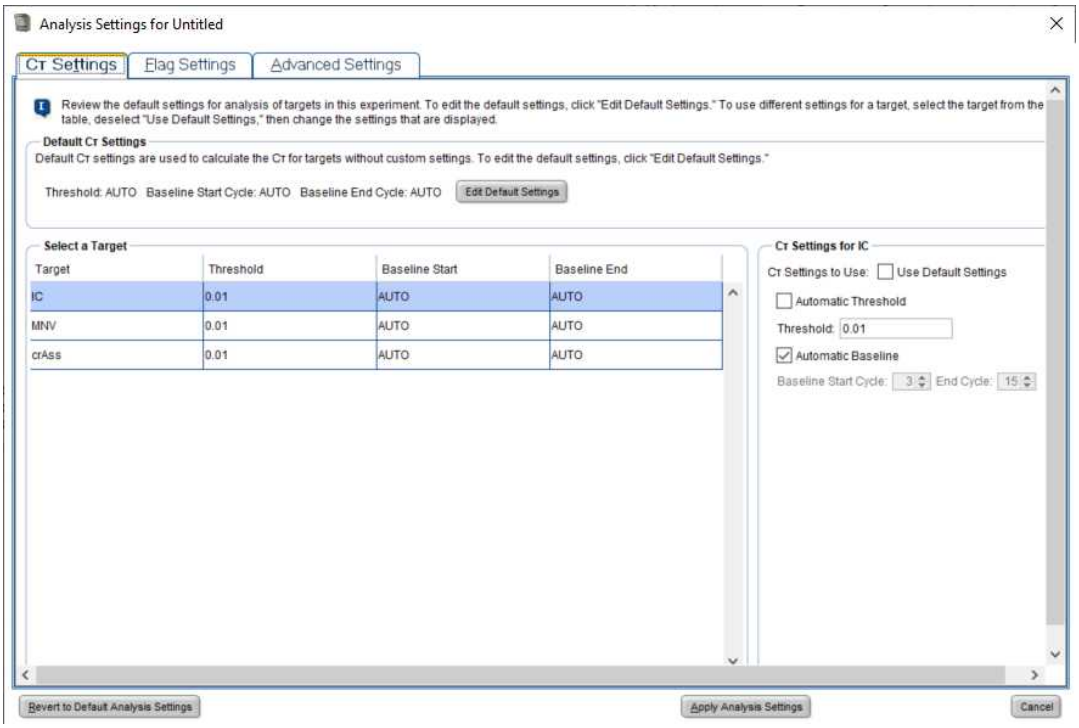
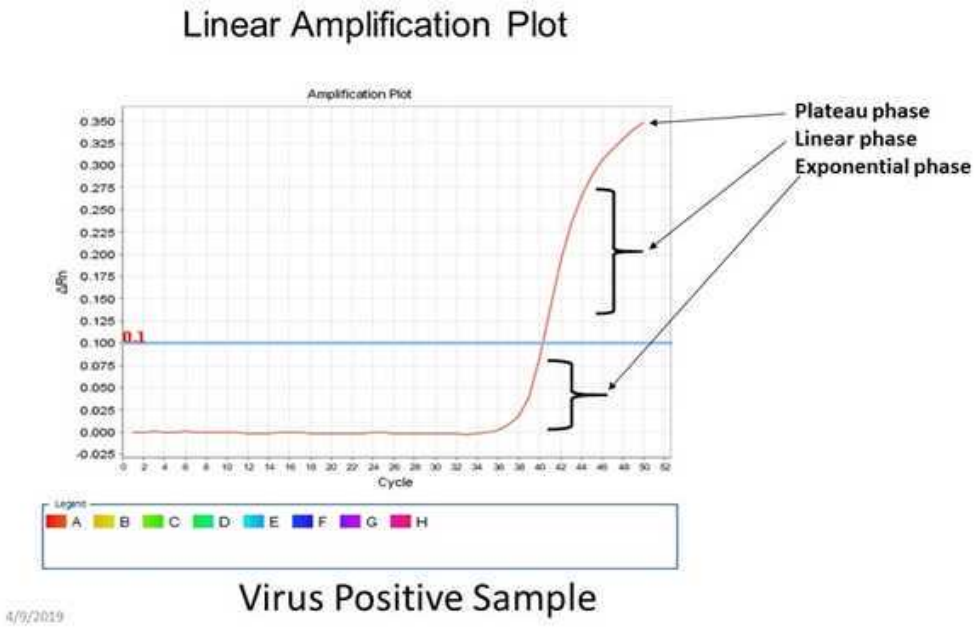


10 Verify positive and negative calls for each reaction using either linear or log amplification plots.

10.1



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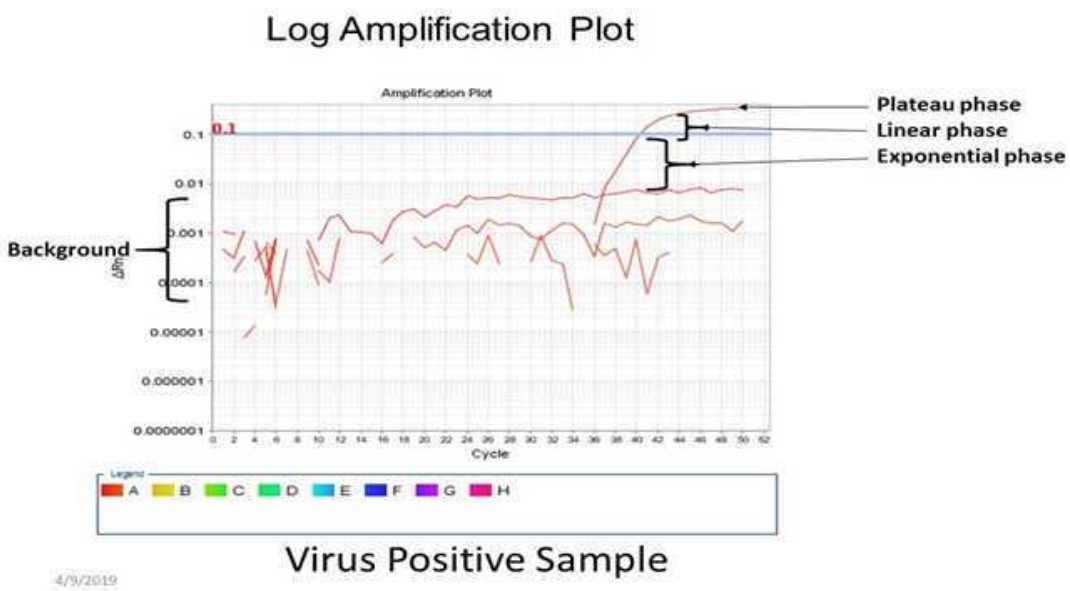
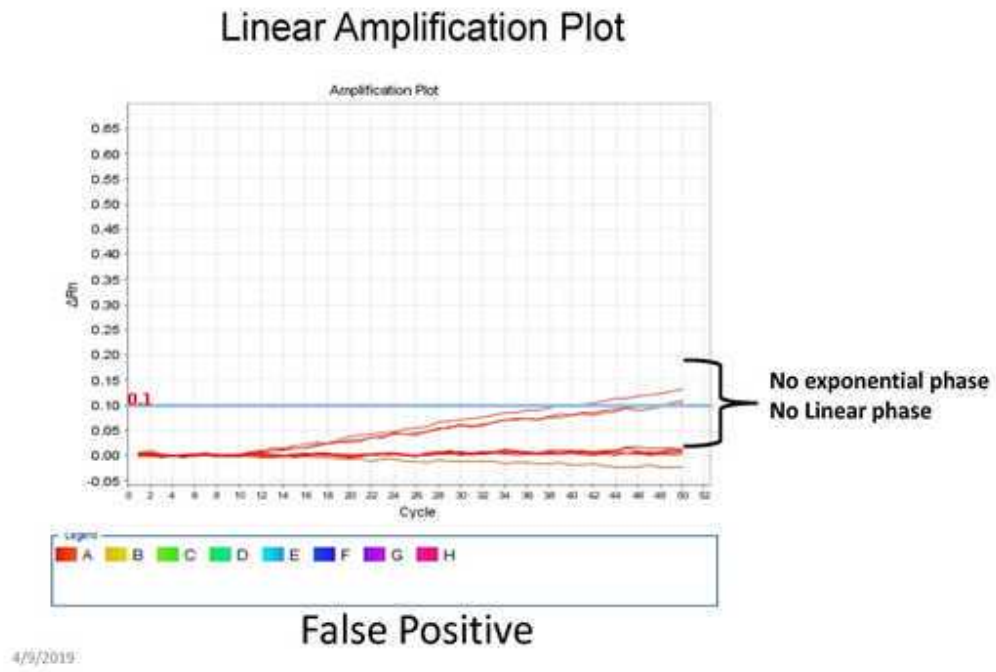
10.2



10.3



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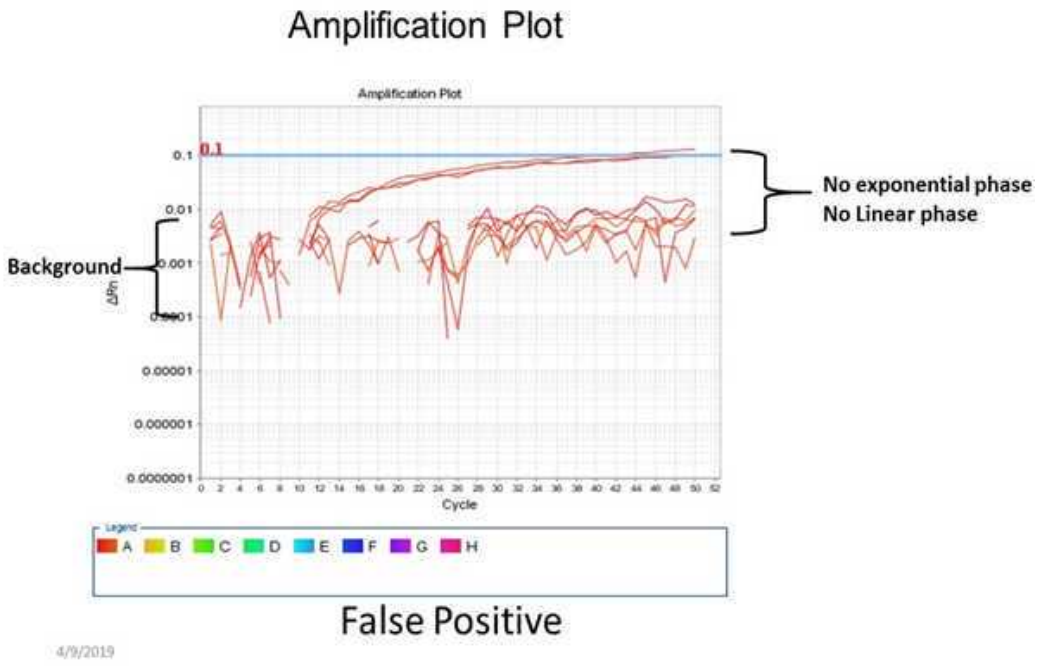
10.4



11 Sample is **invalid** if *any* of the following are observed:

1. Negative RT-qPCR control is positive (Ct value indicated) for any of the expected targets (MNV or crAssphage);
2. Positive RT-qPCR control is negative (undetermined) for expected target/s (MNV or crAssphage);
3. Sample is negative (undetermined) for MNV; *or*
4. IC is negative (undetermined) in the sample, or the average of the IC Ct values from the sample replicates are greater than 4 Cts than the IC Ct of the negative RT-qPCR control.

Sample is **invalid** and RT-qPCR should be repeated. If still invalid, an additional concentrate from Virus Concentration from Wastewater Using PEG Precipitation and Ultracentrifugation (protocols.io) should be extracted and tested.

12 Sample is **valid** if *all* of the following are observed:

1. Negative RT-qPCR control is negative (undetermined) for expected target/s (i.e., MNV and crAssphage);
2. Positive RT-qPCR control is positive (Ct value indicated) for expected target/s (i.e., MNV and crAssphage); *and*
3. Internal amplification control (IC) is positive in all sample reactions with the average IC Ct values for the sample is less than 4 Cts greater than the IC Ct of the negative RT-qPCR control.

Sample is **valid**, proceed to determination of SARS-CoV-2 results as described RT-qPCR Detection of SARS-CoV-2 from Wastewater Using the AB 7500 (protocols.io).

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