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Dec 02, 2021

RT-qPCR Detection of SARS-CoV-2 from Wastewater Using the AB 7500 V.1

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1

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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 triplex and duplex assays for the RT-qPCR detection of the nucleocapsid region of the SARS-CoV-2 genome. These assays, along with the murine norovirus (MNV; extraction control) and crAssphage (human indicator) RT-qPCR assay (RT-qPCR Detection of Process Controls (Murine noroviurs and crAssphage) from Wastewater (protocols.io)), were developed for use on the AB 7500 platform using software version 2.0 or 2.3. All assays incorporate an internal amplification control (IC) to prevent the reporting of false negatives due to inhibition or failure of the RT-qPCR. These multiplexed detection assays were developed for the qualitative determination SARS-CoV-2 nucleocapsid gene extracted from wastewater. Valid sample results are contingent upon the detection of the MNV extraction control from the sample being tested.

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GenomeTrakr, wastewater, SARS-CoV-2, N gene, 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) or ice bucket with ice
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:

☒ SARS-CoV-2 Genomic RNA **ATCC Catalog #1992D**

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

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

☒ 50mM MgCl2 **Bio-rad Laboratories Catalog #17088872** or

☒ 25 mM MgCl2 **Thermo Fisher Scientific Catalog #AB0359**

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

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

☒ FAM reference dye **Bio-rad Laboratories Catalog #1708780**, or equivalent, if running the N1-N2-IC triplex assay

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ROX reference dye **Fisher Scientific Catalog #12-223-012** , if running the N1-IC and N2-IC duplex assays

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**

Primers and probe sequences in following table.

Custom Primers and Probes **IDT Technologies** .

Identification	Primers (Sequence 5' -> 3')	Location#
N1F[a]	ACCCCAAAATCAGCGAAAT	28288-28306
N1R	CTGGTTACTGCCAGTTGAATCTG	28357-28335
N2F	TTACAAACATTGGCCGCAA	29164-29182
N2R	CGCGACATTCCGAAGAA	29229-29213
IC46F[b]	GACATCGATATGGGTGCCG	N/A
IC194R[b]	AATATTCGCGAGACGATGCAG	N/A
Identification	**Probes**	**Location#**
N1P[c]	FAM or JOE- ACCCCGCATTACGTTTGGTGGACC -IB-RQ*	28309-28332
N2P[c]	FAM or Cy5- ACAATTTGCCCCCAGCGCTTCAG -IB-RQ*	29188-29210
IACP[b]	TxRed- CCTTCCCGACCGATGGCATC -IB-RQ*	N/A

#based on accession nos. JF320650, MT006214.1, and NC_045512
[a]Luet al., 2020
[b]Depaola, Jones, Woods et. al. 2010, U.S. Patent Application 0060166232
[c]Fluorophorefor N1 and N2 should be FAM when running duplex assays, or JOE and Cy5, respectively, if running the triplex assay
*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.

Detection of SARS-CoV-2 can be conducted as a triplex assay (N1-N2-IAC), or as two

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duplex assays (N1-IAC and N2-IAC).

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 5s

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 Detection of SARS-CoV-2 from Wastewater (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 Tables for SARS-CoV-2 Assays .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] or ROX[B] dye	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.
[B] Use a 1:10 dilution (made in Primer TE) of ROX reference dye in the N1-IC and N2-IC duplex assays.

*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).

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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.

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.

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

Reaction Set-Up 5s

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. 5s

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** . 5s

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)"

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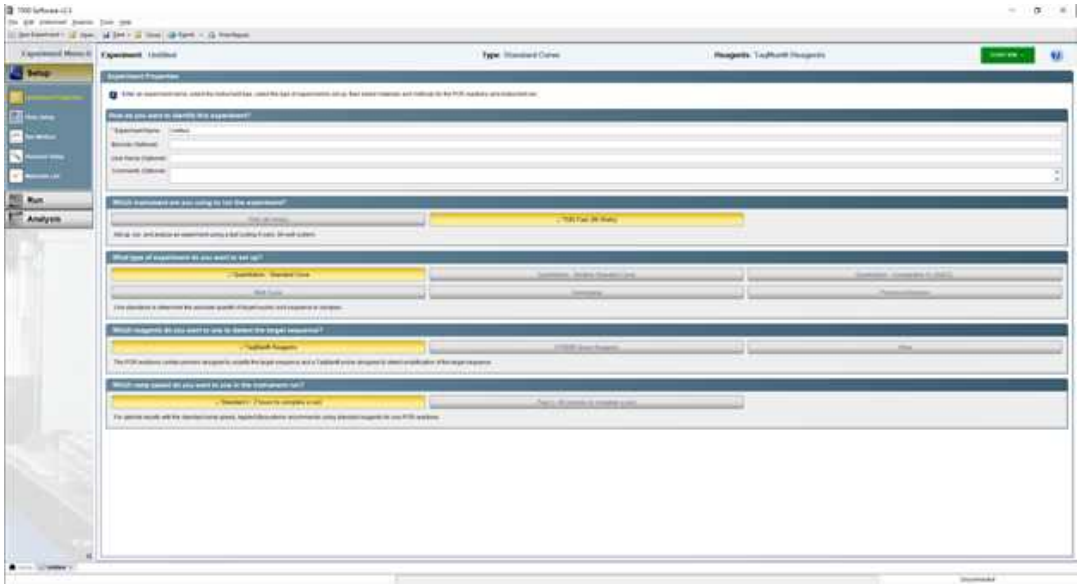
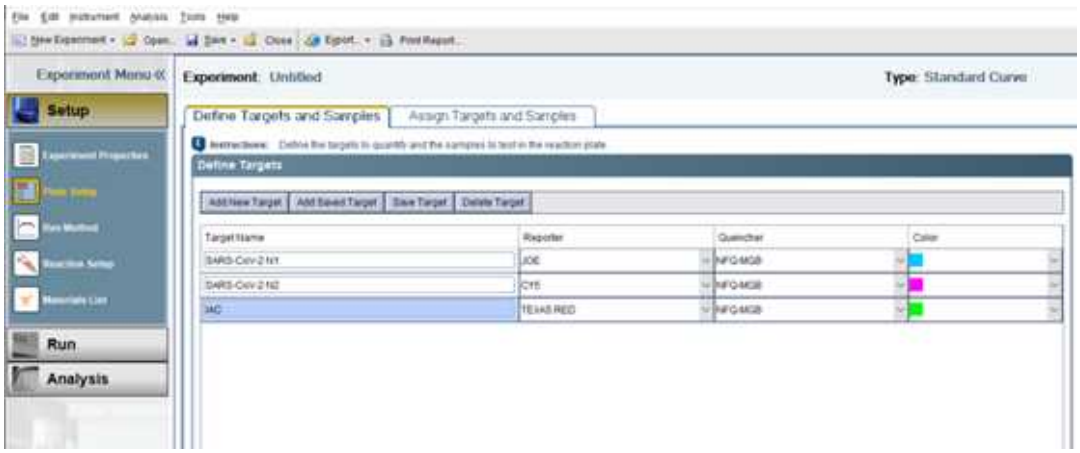
"Quantitation Standard Curve"
"TaqMan Reagents"
"Standard (~2 hours to complete run)"



8.2 Identify the appropriate target reporters and leave all quenchers as "NFQ-MGB".



Example in image is for the N1-N2-IAC triplex assay (N1-Joe, N2-Cy5, IC-Texas Red). If using the duplex assays, adjust appropriately (N1-FAM, N2-FAM, IC-Cy5).

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

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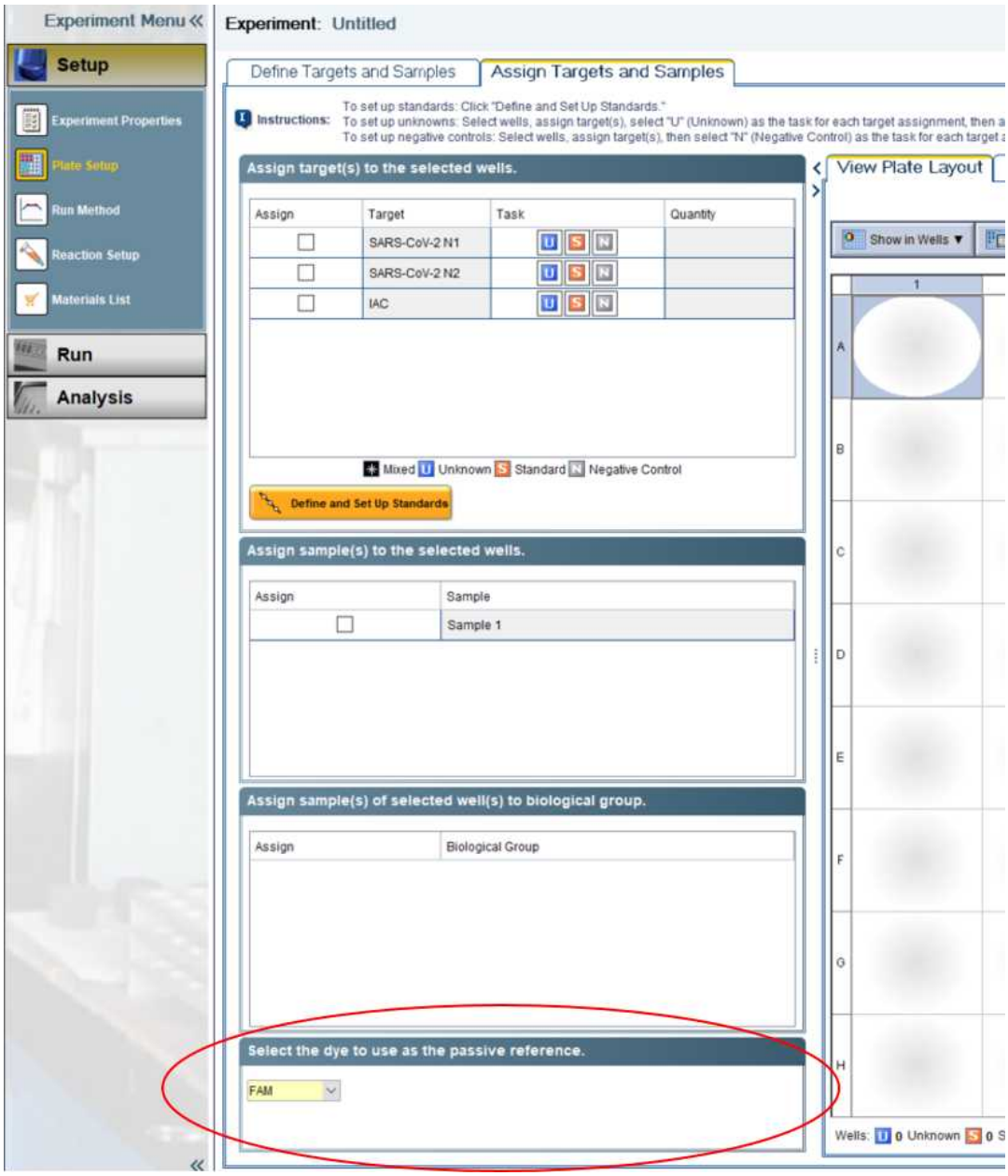

Example in image is for the N1-N2-IAC triplex assay. If using the duplex assays, adjust appropriately (ROX).

8.4 Assign targets and samples.

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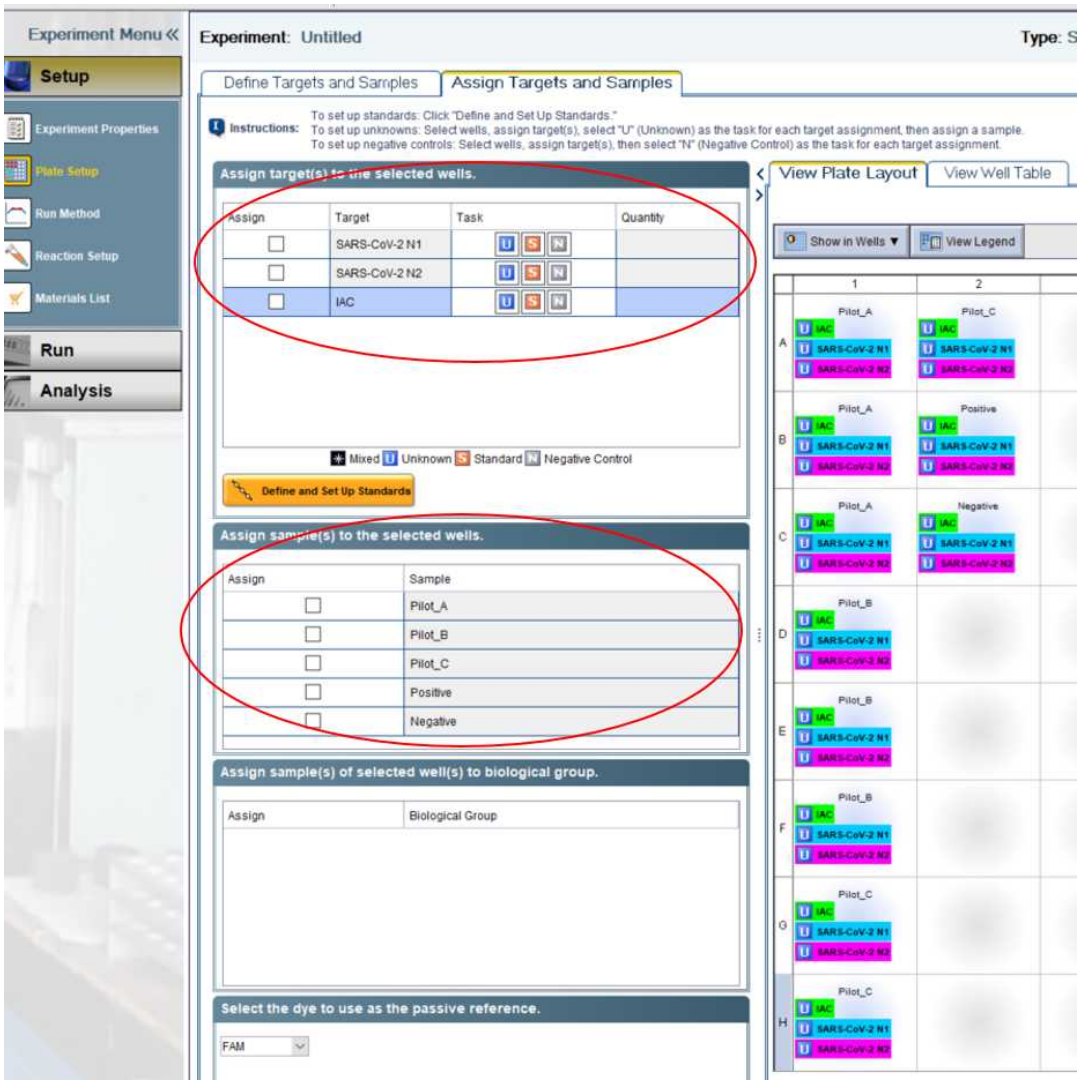

1h 6m 35s

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 **00:00:10**, **56 °C** for **00:00:25**, **62 °C** for **00:01:00**

Enable data collection on Step 3 of Cycling stage

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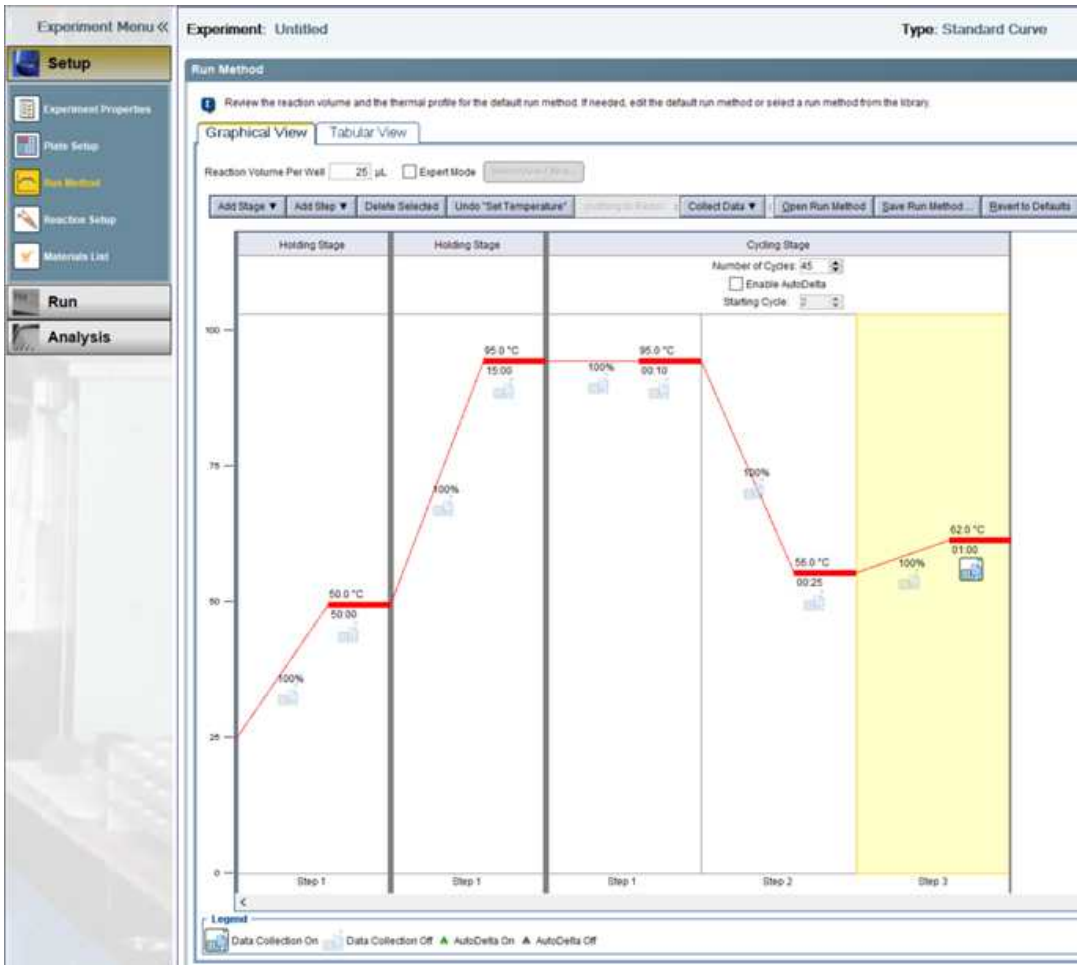

These are the cycling conditions for the N1-N2-IAC triplex as well as the N1-IAC and N2-IAC duplex assays.

Data Analysis

9 Adjust analysis settings to appropriate thresholds. For the triplex assay, N1 and N2 should be set at **0.01** and the IC set at **0.1**. Baseline start cycle should be set at 3 and baseline end cycle should be set at 10.

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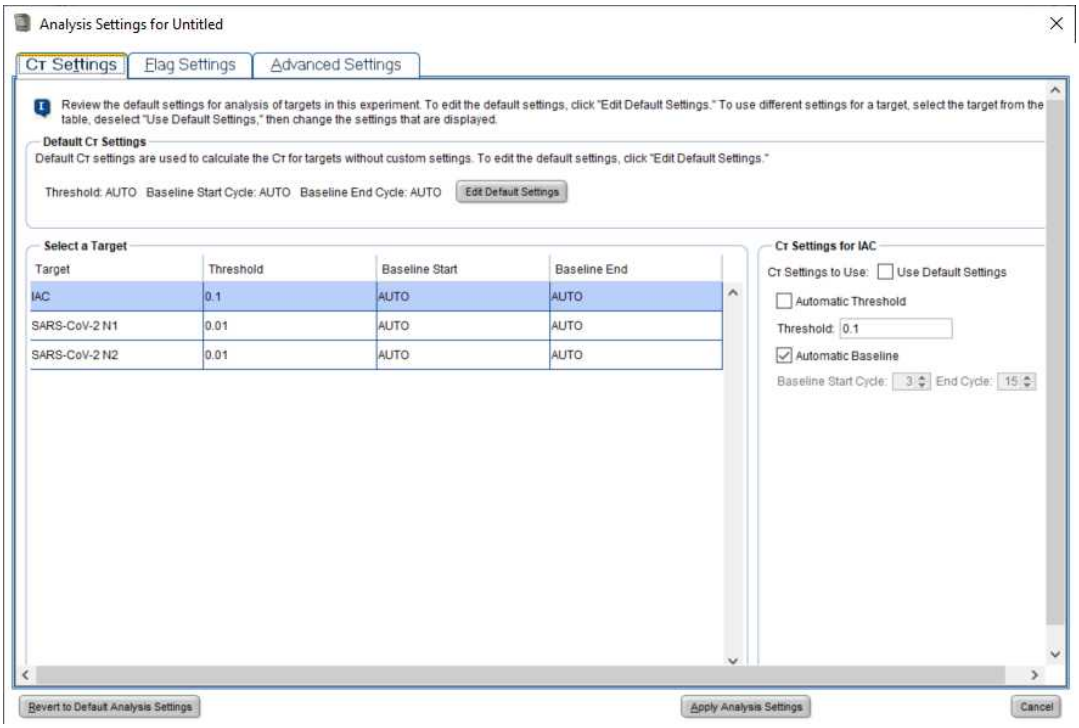

Example in image is for the N1-N2-IAC triplex assay. If using the duplex assays, adjust appropriately (all thresholds set at **0.01**).

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

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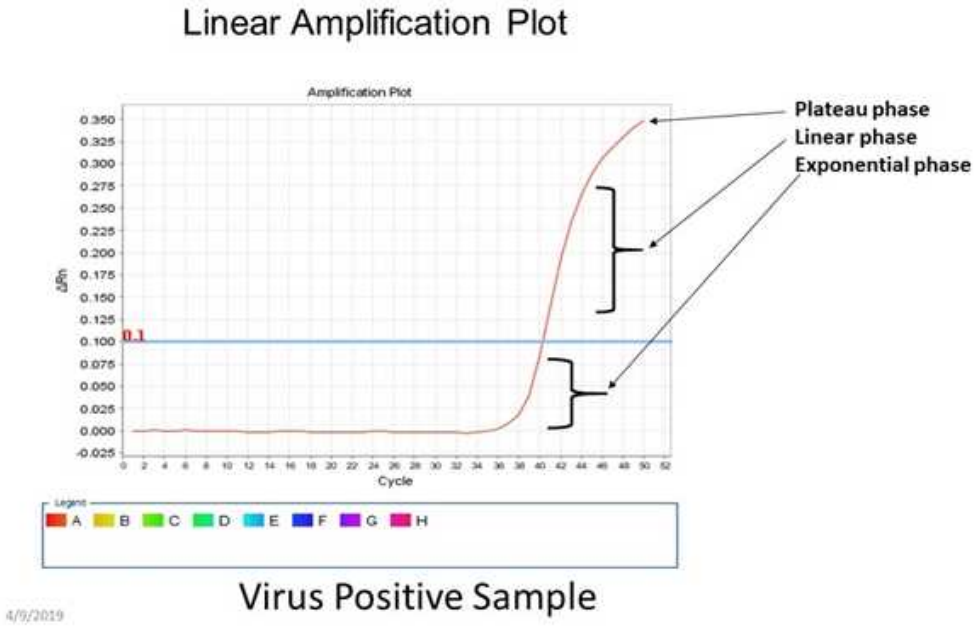
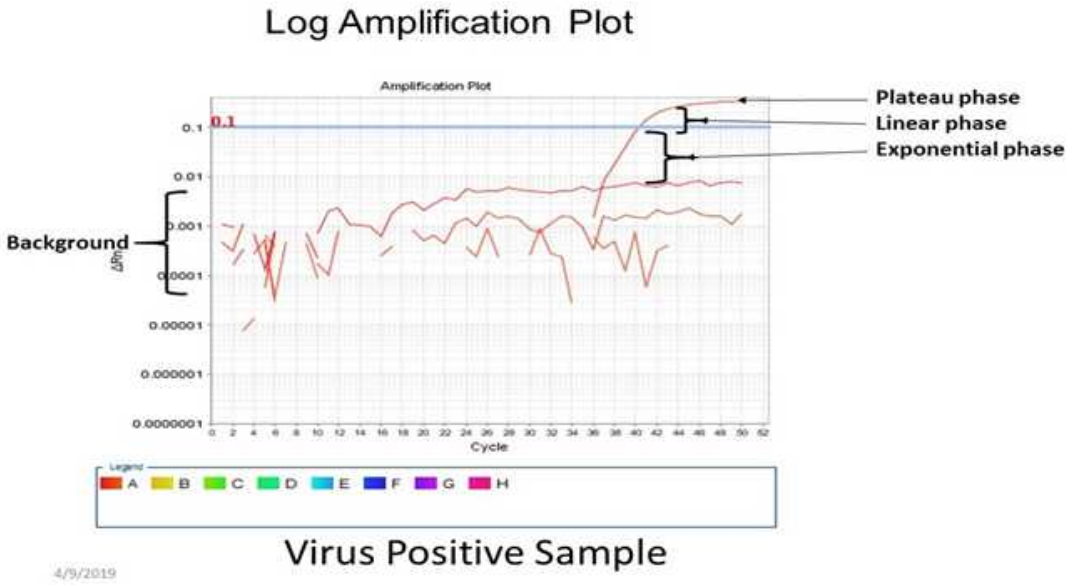
10.1



10.2



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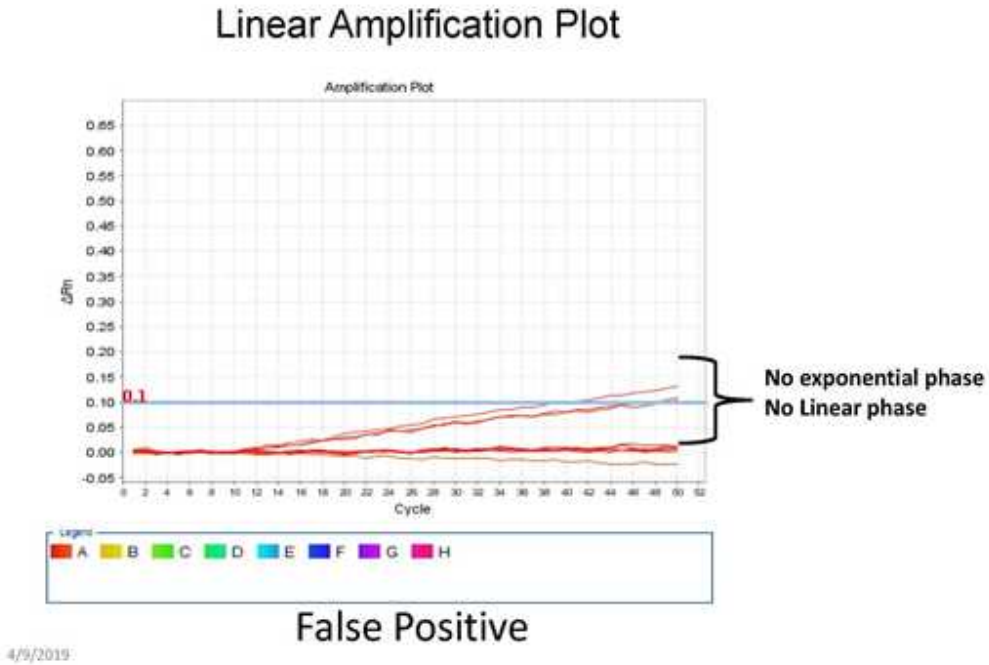
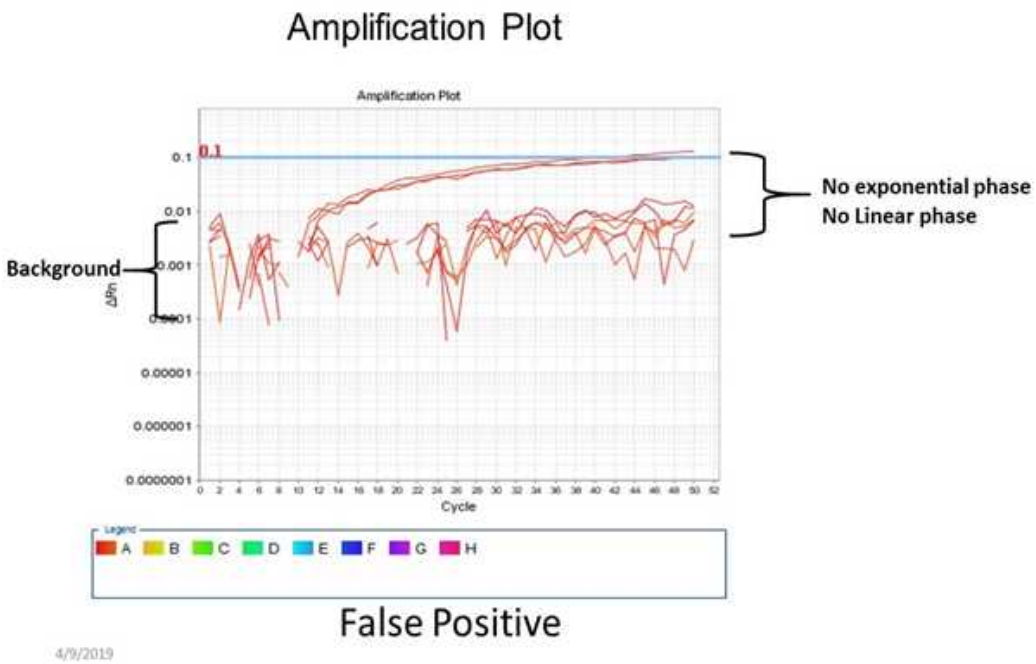
10.3



10.4



11 RT-qPCR run is **invalid** if *any* of the following are observed:

1. Negative RT-qPCR control is positive (Ct value indicated) for any of the expected SARS-CoV-2 targets;
2. Positive RT-qPCR control is negative (undetermined) for expected SARS-CoV-2 targets; *or*

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3. IC is negative (undetermined) in the negative RT-qPCR control; *or*

Run is **invalid** and the RT-qPCR assay must be repeated.

12 Sample is **negative** if:

1. Negative and positive RT-qPCR control reactions give appropriate results;
2. Sample reaction is negative (undetermined) for expected SARS-CoV-2 target/s; *and*
3. Internal amplification control (IC) is positive.

Valid SARS-CoV-2 results require the successful detection of MNV extraction control from the sample as described in *link protocol*. If MNV is negative for a sample, that sample is **invalid** and should not be reported as negative.

13 Sample is **positive** if:

1. Negative and positive RT-qPCR control reactions give appropriate results; *and*
2. Sample reaction is positive (Ct value indicated) for expected SARS-CoV-2 target/s.

If at least one of the triplicate sample reactions are positive, the sample should be reported as **positive**.

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