RNA extraction is to be performed using manual or automated extraction protocol. The final elution plate containing purified RNA samples is to be used for RT-PCR reaction plate setup. The elution plate is **NOT** to be used directly for the rRT-PCR setup. Perform master mix and reagent setup in a new PCR plate.

RT-PCR Run Setup

1. In the Bio-Rad CFX Maestro home screen, select the **Run Setup** tab. Enter or confirm instrument as the instrument to be used. Select run type as **User-defined**.
2. In the Protocol tab, click **Select Existing…** and navigate to the AzureSeqPlus protocol template.
    - For reference, see thermocycling protocol below (20μL reaction volume).

Step	Temperature	Time	Number of Cycles
RT Incubation	50°C	15 minutes	1
Enzyme Activation	95°C	2 minutes	1
Amplification	95°C	3 seconds	40-45
	60°C**	30 seconds	

**collect fluorescence during annealing/extension phase (60°C) step on all four channels.

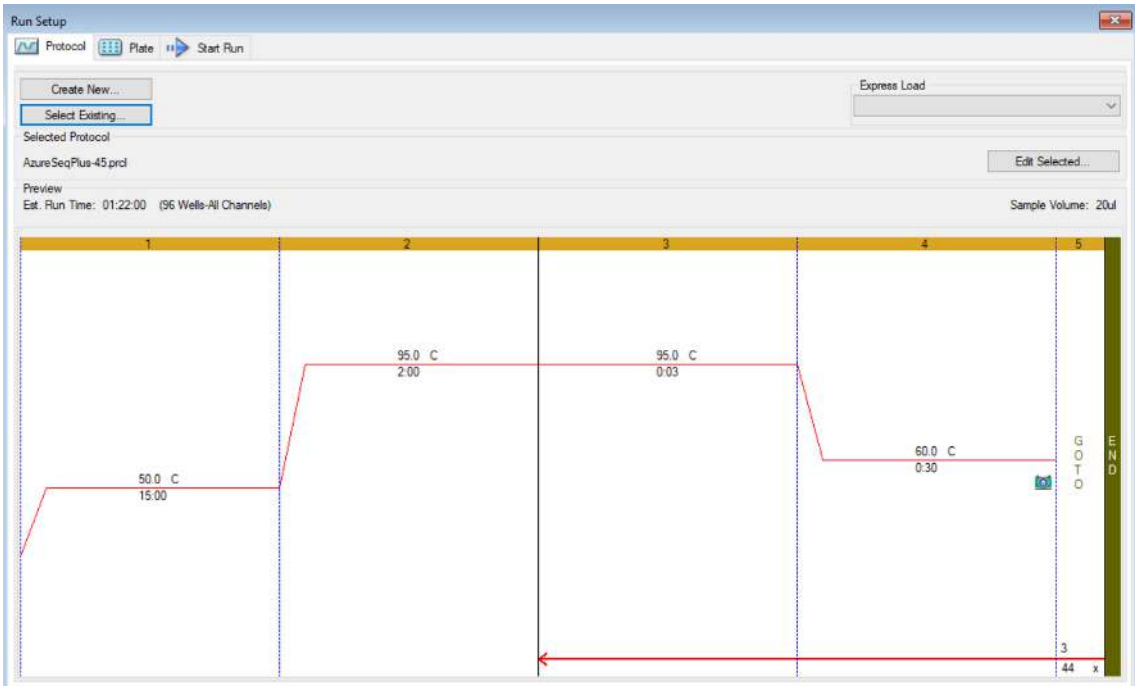

Figure 1: Run setup – protocol

3. In the Plate tab, click **Select Existing…** and navigate to the AzureSeqPlus plate template.
4. In the toolbar, select **Spreadsheet View/Importer**, then add in sample names per well or import a sample list.
5. In the **Define** tab for Targets, confirm that the targets and reporter dyes are listed correctly.

- Confirm all applicable wells' Sample Type as **Unknown** (except for each control), all with the following targets and dyes.

Target	Reporter Dye
SARS-CoV-2 N1	FAM
Influenza B	HEX (can also be VIC or JOE)
RNaseP	Texas Red (can also be ROX)
Influenza A	Cy5 (can also be Quasar 670)

6. Verify plate layout has samples/wells designated for control samples. Positive control, negative control, and no template control (PCR blank) should be present for each run.

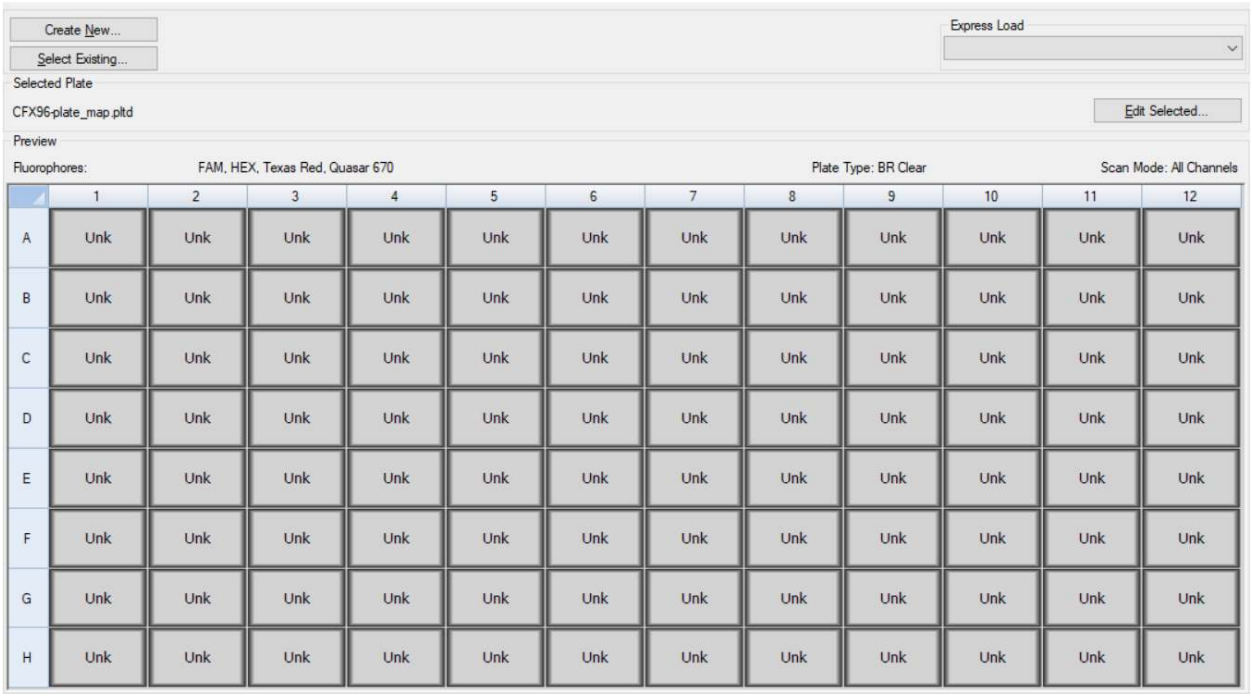

Figure 2: Run setup - plate

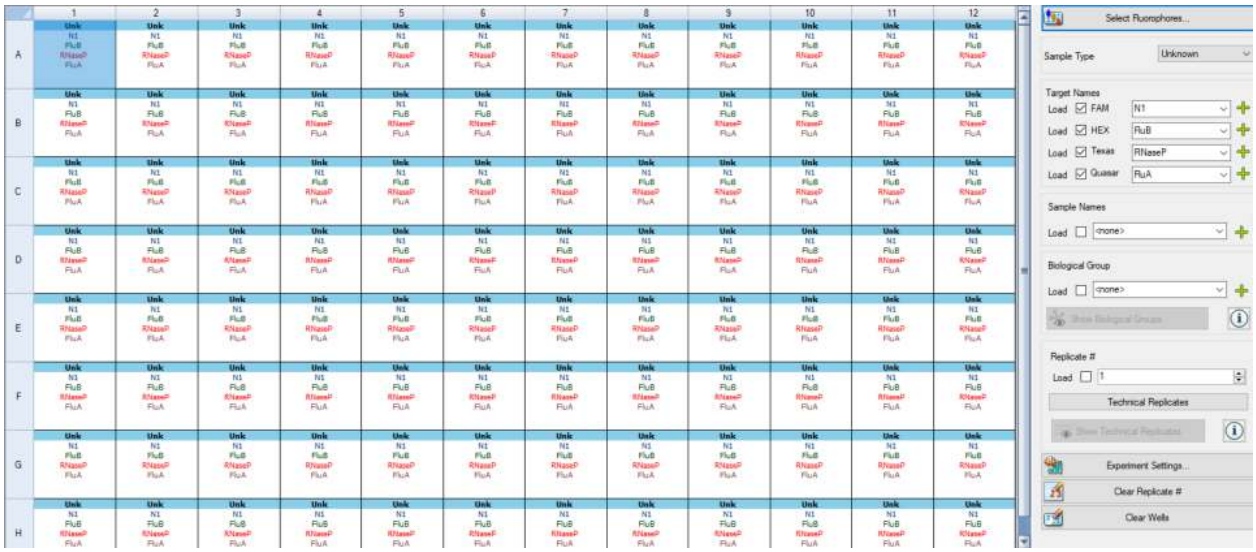

Figure 3: Detailed plate setup

RT-qPCR Master Mix and Reagent Setup

1. Completely thaw the 2x InhibiTaq MM and CoVi Primer/Probe mix 4 (brown tube/green cap) by setting on ice for ~30 minutes. Reagents can be thawed at room temperature for ~5 minutes. Once thawed, briefly centrifuge to collect at the bottom of the tube.
2. Vortex the reagent tubes at max speed for 10 seconds to mix, then spin down briefly to collect at bottom of tube.
3. Proceed to combine in a 1.5mL (15mL conical tube for 384-well format) Eppendorf tube, in order shown below, in a clean room or designated setup area.

Component	Volume 1x Reaction (µL)	Volume 100 reactions (96-well plate (µL))	Volume 400 reactions (384-well plate (µL))
Sterile Water	2.75	275	1100
CoVi Primer/Probe Mix 4	1	100	400
2x InhibiTaq Multiplex qPCR	10	1000	4000
RTScript™, 200U/µL	1.25	125	500

4. Mix the master mix by pipetting up and down repeatedly or by capping the tube, vortexing briefly, and spinning down briefly to collect mix.
5. Pipette 15µL of the master mix to all wells of the 96- or 384-well PCR plate that is on a cold block.
6. Pipette 5µL of sample, positive control, or negative control to the appropriate wells of the 384-well PCR plate.
7. Pipette 5µL of nuclease-free water into the well designated as no template control (PCR blank).

8. Seal the plate, vortex briefly, then spin down in a centrifuge to collect the reaction mix.
9. Place the plate in the designated PCR instrument.
10. On the PCR instrument's CFX Maestro software, review the run information that was configured in RT-PCR Run Setup.
11. In the **Start Run** tab, check the box for the applicable instrument and select **Start Run**.
12. Enter a file name in the dialog box that prompts you to save the run file, then save the file.
13. RT-PCR run time is approximately 1h:15m.

Interpretation of Results

1. In the CFX Maestro software after the run is complete, select **File** and **Save As** and select a location to save the run's .pcrd file.
2. Using a USB drive or other method, open the run data file from the computer connected to the instrument to the computer that will be performing the analysis.
3. Open the CFX Maestro software and open the run data file for the completed run.
4. On the toolbar, select **Settings**->**Baseline Setting**->**Apply Fluorescence Drift Correction**.
5. Review each target's threshold by selecting the target with the checkboxes between the amplification plot and the plate layout. Ensure each target's threshold is above the background while intersecting the reaction curves towards the beginning of the exponential phase.
    a. To modify, select the baseline and adjust as necessary.

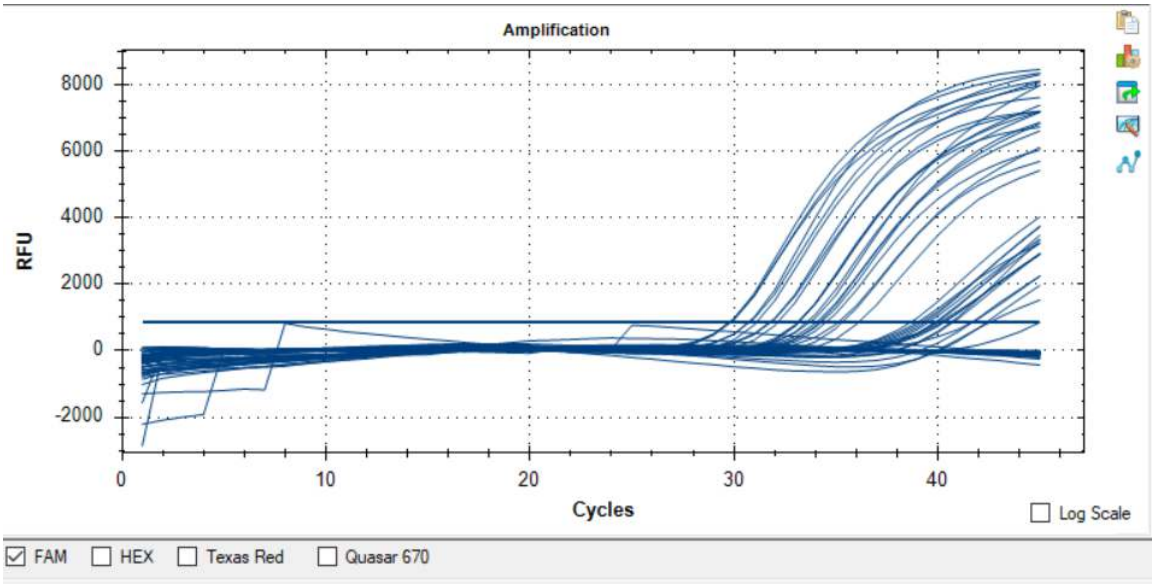

Figure 4: Single fluorophore amplification plot with threshold

6. Review positive/negative/no template control samples to ensure their validity before continuing with the run analysis. If controls are not valid, the patient results cannot be interpreted.

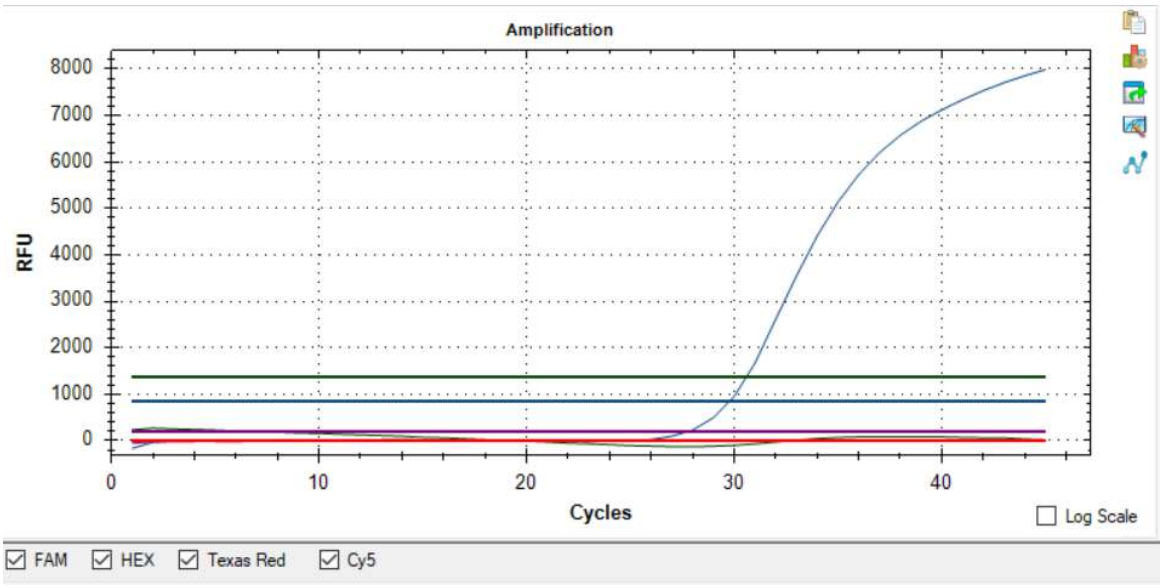

Figure 5: Positive SARS-CoV-2 sample amplification plot

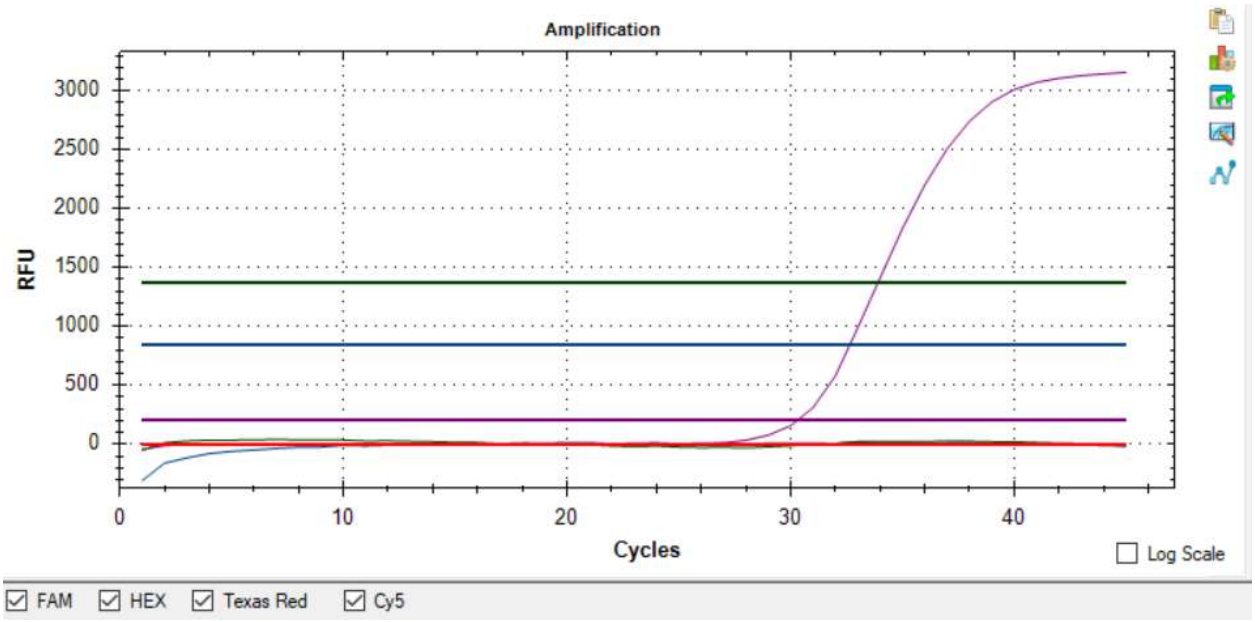

Figure 6: Positive Influenza A sample amplification plot

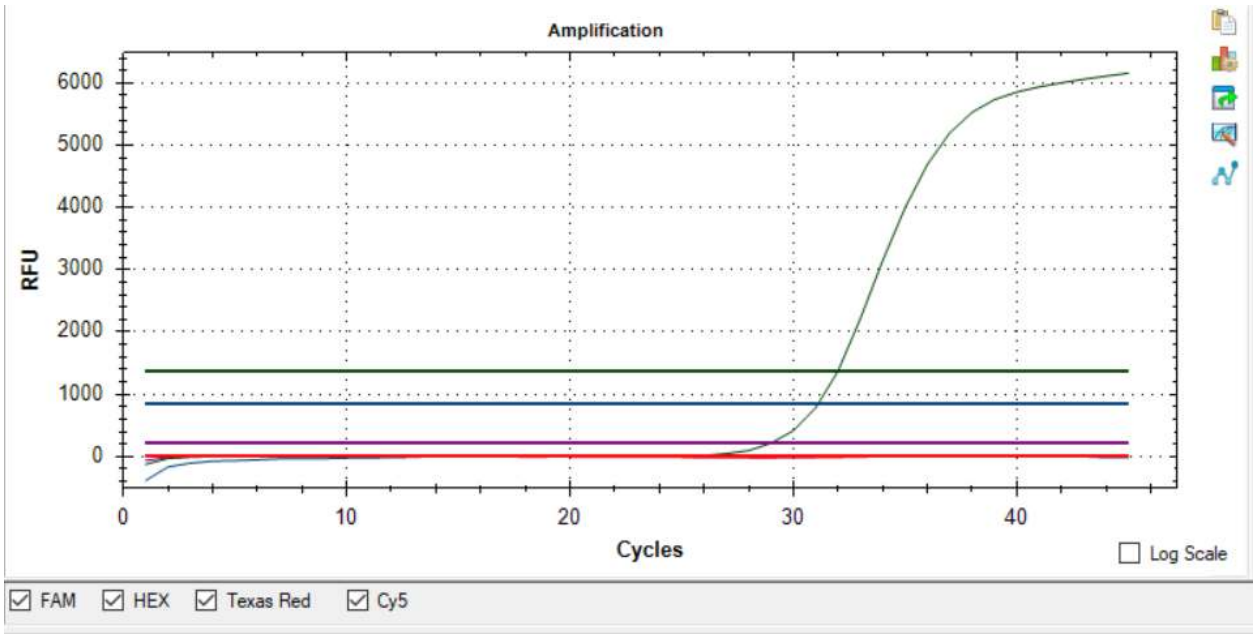

Figure 7: Positive Influenza B sample amplification plot

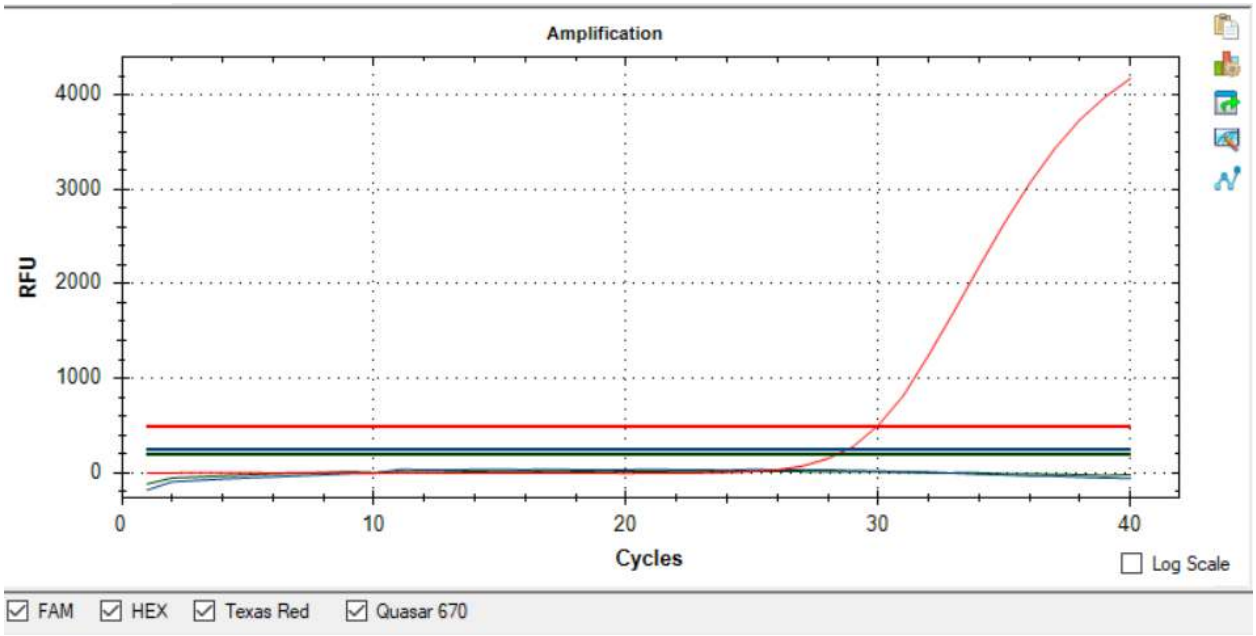

Figure 8 Negative sample amplification plot

7. (If making interpretations by Ct review), begin review of run data using the following chart with a Ct cutoff of 40.

SARS-CoV-2 (FAM)	Influenza A (Qua670)	Influenza B (HEX)	RNaseP (ROX)	Result Interpretation	Action
+	-	-	+/-	Positive SARS-CoV-2	Report results to the healthcare provider and appropriate public health agencies
-	-	-	-	Invalid	Repeat test. If still invalid collect another specimen. If another specimen is not available, report to the healthcare provider
-	-	-	+	SARS-CoV-2, Influenza A, Influenza B not detected	Report results to healthcare provider
-	+	-	+/-	Positive Influenza A	Report results to the healthcare provider and appropriate public health agencies
-	-	+	+/-	Positive Influenza B	Report results to the healthcare provider and appropriate public health agencies
+	+	-	+/-	Positive SARS-CoV-2 Positive Influenza A	Report results to the healthcare provider and appropriate public health agencies
-	+	+	+/-	Positive Influenza A Positive Influenza B	Report results to the healthcare provider and appropriate public health agencies
+	-	+	+/-	Positive SARS-CoV-2 Positive Influenza B	Report results to the healthcare provider and appropriate public health agencies
+	+	+	+/-	Positive SARS-CoV-2 Positive Influenza A Positive Influenza B	Report results to the healthcare provider and appropriate public health agencies

8. Review any non-Sigmoidal or other non-standard amplification curves and downgrade/upgrade result interpretation as necessary.
9. After all samples have been reviewed, select **Export** -> **Custom Export** to export run data for LIS import.

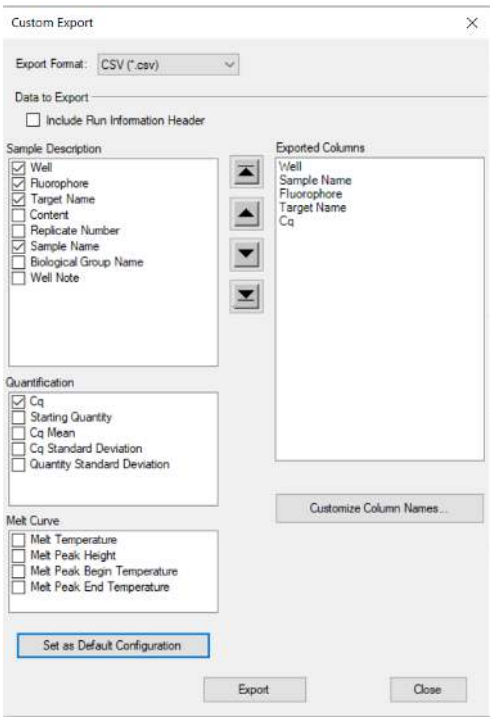

Figure 9: Custom Export configuration