Agilent SARS-CoV-2 qRT-PCR Dx Kit
K1180A
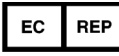
Instructions for Use

For In Vitro Diagnostic Use
For Export Only. Not for Sale in the United States.

Revision E0, April 2021

Symbol Table

Symbol	Meaning	Symbol	Meaning
CE	European Conformity	⚠	Caution
IVD	In Vitro Diagnostic Medical Device	REF	Catalog/code number
🏭	Manufacturer	📖	Consult Instructions for Use
⧖	Use-by date	Σ▽	Contains sufficient for <N> tests
LOT	Batch code	⊘	Do not reuse
🌡	Temperature limitation	EC REP	Authorized representative in the European Community

Notice to Purchaser: Limited License

This product is sold under licensing arrangements between Agilent and Life Technologies Corporation. The purchase price of this product includes limited, nontransferable rights owned by Life Technologies Corporation to use only this amount of the product for use in *in vitro* testing of SARS-CoV-2 in humans in accordance with the instructions for use accompanying this product. No other rights are conveyed. Further information on purchasing licenses under the above patent may be obtained by contacting the Licensing Department, Life Technologies Corporation, 5781 Van Allen Way, Carlsbad, CA 92008. Email: **outlicensing@lifetech.com**.

6   Analysis and Results

7   Quality Control

8   Limitations of the Assay

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1 Agilent SARS-CoV-2 qRT-PCR Dx Kit Product Information

This chapter contains introductory information on the assay.

Intended Use

Agilent SARS-CoV-2 qRT-PCR Dx Kit is a real-time RT-PCR in vitro diagnostic test intended for the qualitative detection of RNA from the SARS-CoV-2 isolated and purified from nasopharyngeal (NP), nasal, and oropharyngeal (OP) swab specimens obtained from individuals who meet COVID-19 clinical and/or epidemiological criteria.*

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in upper respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with other clinical observations, patient history, and epidemiological information.

The Agilent SARS-CoV-2 qRT-PCR Dx Kit is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the operation of the Agilent system and in vitro diagnostic procedures.

* The performance of the Agilent SARS-CoV-2 qRT-PCR Dx Kit assay was established using nasopharyngeal swab specimen type collected in UTM or VCM transport media. Oropharyngeal swabs, nasal swabs, and mid-turbinate nasal swabs are considered acceptable specimen types for use with the Agilent SARS-CoV-2 qRT-PCR Dx Kit assay but performance with these specimen types has not been established. Testing of oropharyngeal swabs, nasal swabs, and mid-turbinate nasal swabs (self-collected under supervision of or collected by a healthcare provider) is limited to patients with symptoms of COVID-19.

Product Description

Product Overview/Test Principle

The Agilent SARS-CoV-2 qRT-PCR Dx Kit is a real-time, reverse transcription polymerase chain reaction (qRT-PCR) test. The SARS-CoV-2 primer and probe set(s) is designed to detect RNA from SARS-CoV-2 in nasopharyngeal (NP), nasal, and oropharyngeal (OP) swab specimens from patients suspected of COVID-19 by their healthcare provider.

Description of Test Steps

Nucleic acids are isolated and purified from ~140–200 µl (depending on extraction method) of nasopharyngeal (NP) swab specimens using an automated extraction system (Qiagen QIAsymphony DSP Virus/Pathogen Mini Kit with QIAsymphony, ThermoFisher MagMAX Viral/Pathogen II Kit with KingFisher Flex). The purified nucleic acid (5 µl) is reverse transcribed into cDNA and subsequently amplified in a one-step qRT-PCR reaction using Agilent Brilliant III qRT-PCR reagents on supported real-time PCR instruments. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of *Taq* polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by supported real-time PCR instruments (Agilent AriaMx/AriaDx, ABI 7500 Fast, or Bio-Rad CFX96).

Control Materials to be Used with Agilent SARS-CoV-2 qRT-PCR Dx Kit

- A "no template" (negative) control is needed to check for contamination in the assay process that may lead to false positive results and must be used at least once per PCR plate. The user adds water in place of extracted RNA for use as a no template control.

- A positive template control is needed to ensure the mechanism to detect the targeted SARS-CoV-2 RNA is not compromised and must be used at least once per PCR plate (50 copies/reaction). The positive control in the kit is synthetic RNA corresponding to the viral target sequences.

- An extraction control (Human Specimen Control, or HSC) is needed to ensure RNA in the original specimen is well preserved during extraction and must be used at least once per extraction batch.

- An internal control (Human RNase P gene) is needed to ensure RNA from the original specimen is detectable in the qRT-PCR reaction. It is expected to be detected in the HSC as well as most human specimens with sufficient cellular RNA. In human specimens, the internal control may not be detected if SARS-CoV-2 viral RNA is present at high concentrations.

Controls provided with the kit

Table 1   **Control provided with the Agilent SARS-CoV-2 qRT-PCR Dx Kit**

Type of Control	Name of Control	Supplier's Name	Supplier p/n
Positive	Agilent SARS-CoV-2 Positive RNA Ctl Dx	Agilent	K1180-64200

Controls required but not provided with the kit

- Molecular grade, nuclease-free water, to be used as "no template" (negative) control
- Extraction control: several options are available to the customer based on CDC recommendations
  - Human Specimen Control, manufactured by the CDC, KT0189
  - Negative human specimen material: Laboratories may prepare a volume of human specimen material (e.g., human sera or pooled leftover negative respiratory specimens) to extract and run alongside clinical samples as an extraction control. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.
  - Contrived human specimen material: Laboratories may prepare contrived human specimen materials by suspending any human cell line (e.g., A549, Hela, or 293) in PBS. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.

Assay Workflow

To begin the assay, extract nucleic acid from the nasopharyngeal test specimens alongside a suitable Human Specimen Control.

Next, run a multiplex qRT-PCR experiment using the extracted RNA samples and the provided positive RNA control. Include a No Template Control (NTC).

Upon completion of the qRT-PCR run, analyze the data for amplification of the N1 and N2 viral targets and the human RNase P gene target.

Finally, report the results for the test specimens.

```
┌─────────────────────────────────────────┐
│   Sample lysis, nucleic acid capture, and│
│   elution transfer using test specimens  │
│   and extraction control                 │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│   qRT-PCR with test specimen RNA and     │
│   control reactions for multiplex        │
│   detection of targets N1, N2, and RNase P│
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│   Analysis of qRT-PCR data to determine  │
│   which targets are present in each test │
│   and control sample                     │
└─────────────────────────────────────────┘
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                    ▼
          ┌──────────────────┐
          │  Report results  │
          └──────────────────┘
```
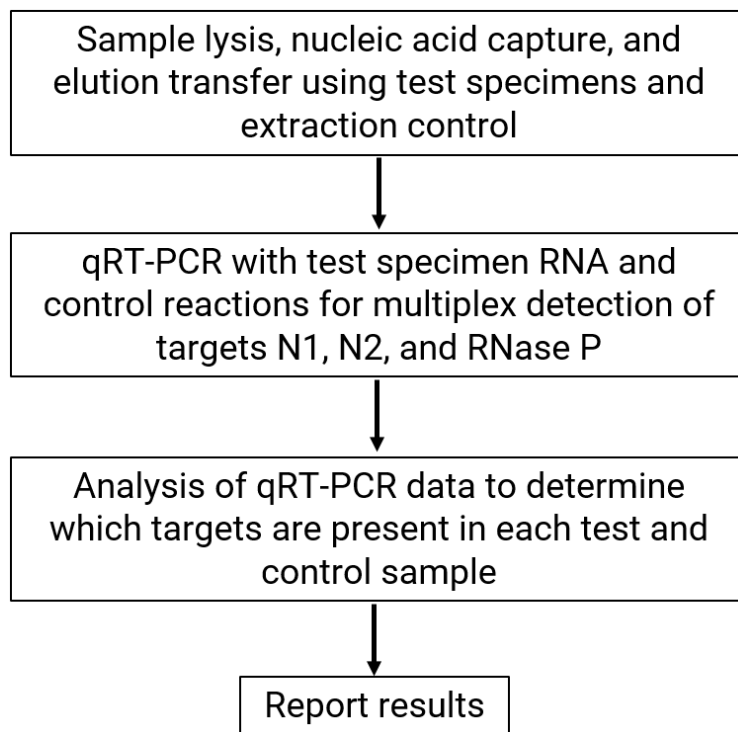
Figure 1   Workflow of the SARS-CoV2 qRT-PCR Dx Assay

2 Materials, Safety, and Handling

This chapter describes the reagents and other materials used in the assay and provides information for safely performing the assay.

Materials Provided

Table 2 lists the materials provided with the Agilent SARS-CoV-2 qRT-PCR Dx Kit and their temperature requirements. See **"Storage and Handling"** on page 17 for additional instructions on storing the materials.

Table 2   Materials provided with Agilent SARS-CoV-2 qRT-PCR Dx Kit, p/n K1180A

Materials	Quantity	Components	Storage Temperature
Agilent SARS-CoV-2 qRT-PCR Dx Reagents, p/n K1180-64100*	Sufficient for 400 qRT-PCR reactions (test specimens and controls) *Note that each qRT-PCR reaction plate must include at least 3 wells for control reactions.*	2× Brilliant III Ultra-Fast qRT-PCR Master Mix Dx	Store at −20°C upon receipt.
		RT/RNase Block Dx	Store at −20°C upon receipt.
		Reference Dye Dx[†]	Store at −20°C upon receipt.
		100 mM DTT Dx	Store at −20°C upon receipt.
		10× SARS-CoV-2 Primer/Probe Mix Dx[†]	Store at −20°C upon receipt.
Agilent SARS-CoV-2 Positive RNA Ctl Dx, p/n K1180-64200[‡]	Sufficient for 8 assay tests	SARS-CoV-2 Synthetic Positive RNA Control Dx	Store at −80°C upon receipt.

* The SARS-CoV-2 qRT-PCR Dx Reagents kit is also sold separately as Agilent p/n K1180B.
[†] This reagent is light sensitive and must be kept away from light whenever possible.
[‡] The SARS-CoV-2 Positive RNA Ctl Dx is also sold separately as Agilent p/n K1180C.

Reagents, Materials, Equipment, and Software Required But Not Provided

Table 3 lists the options for the RNA extraction step of the protocol, which is performed on an instrument for automation. The RNA extraction procedure is described in **"Instructions for Extracting Nucleic Acids"** on page 18.

Table 3   RNA extraction options - Required but not provided

RNA Extraction Kit	Instrument
QIAGEN QIAsymphony DSP Virus/Pathogen Midi Kit, p/n 937055	QIAGEN QIAsymphony SP, p/n 9001297 (automation)
Thermo Fisher Scientific MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit, Thermo Fisher p/n A48383	Thermo Fisher Scientific KingFisher Flex Purification System, p/n 5400630 (automation)

Table 4 lists the options for the Human Specimen Control. This control sample is a human cell culture preparation used as an extraction procedural control to demonstrate successful recovery of nucleic acid, as well as extraction reagent integrity.

Table 4   Human Specimen Control options - Required but not provided

Human Specimen Control	Description
Human Specimen Control (HSC), 10 vials × 500 μl, CDC p/n KT0189	Manufactured and sold by the CDC. The CDC HSC consists of noninfectious (beta-Propiolactone treated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2–7.4.
Negative human specimen material	Prepared by the laboratory. This type of Human Specimen Control is a volume of human specimen material (e.g., human sera or pooled leftover negative respiratory specimens) to extract and run alongside clinical samples as an extraction control. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.
Contrived human specimen material	Prepared by the laboratory. This type of Human Specimen Control is contrived human specimen materials, prepared by suspending any human cell line (e.g., A549, Hela or 293) in PBS. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the Human Specimen Control listed in these Instructions for Use.

Table 5 through **Table 6** list additional reagents, materials, equipment, and instrument that are required but not provided with the Agilent SARS-CoV-2 qRT-PCR Dx Kit.

Table 5   Reagents and materials required but not provided

Reagent or material	Form
Molecular grade, nuclease-free water	Reagent
10% bleach solution (1:10 dilution of 5.25−6% hypochlorite bleach)	Material
DNA*Zap*, Ambion p/n AM9890, or equivalent decontamination agent	Material
RNase AWAY, Fisher Scientific p/n 21-236-21, or equivalent decontamination agent	Material
Disposable powder-free gloves and surgical gowns	Material
Sterile, nuclease-free, aerosol-barrier pipette tips	Material
Nuclease-free 1.5-ml microcentrifuge tubes	Material
Nuclease-free 2-ml microcentrifuge tubes	Material
Nuclease-free 96-well plates, 200-μl *Use plates that are compatible with the selected real-time PCR system*	Material
Nuclease-free 8× tube strips	Material
Nuclease-free adhesive plate seals, *or* Nuclease-free 8× optical cap strips for 96-well PCR plates *Use seals or strips that are compatible with the selected real-time PCR system*	Material
MicroAmp Optical Film Compression Pads, Thermo Fisher Scientific, p/n 4312639*	Material

* The compression pads are only needed when using adhesive seals to seal plates for the AriaMx/AriaDx Real-Time PCR system

Table 6   Instruments, software, and equipment required but not provided

Instrument, software, or equipment	Form

Real-time PCR system
• Agilent AriaMx Real-Time PCR System (p/n G8830A) or AriaDx Real-Time PCR System (p/n K8930AA)
   Software: Aria software version 1.71 or 1.8, and Electronic Tracking Software (included with p/n K8930AA or available separately as p/n G5380AA)
• ABI 7500 Fast Real Time PCR Instrument with laptop computer (p/n 4351106) or desktop computer (p/n 4351107)
   Software: 7500 Software version 2.3 and Design & Analysis Software version 2.4.3
• Bio-Rad CFX96 Touch Real-Time PCR Detection System (p/n 1855195) or CFX96 Touch Real-Time PCR Detection System with Starter Package (p/n 1855196)
   Software: CFX Maestro Software 2.0 version 4.1.2 (included with p/n 1855196 or available separately as p/n 12004110)
	Instrument with computer and software

Software for viewing data exported from the real-time PCR software, e.g., Microsoft Excel	Software
Vortex mixer	Equipment
Microcentrifuge	Equipment
Plate centrifuge for 96-well plates	Equipment
P10, P20, P200 and P1000 micropipettors	Equipment
Multi-channel pipettors (5−50 μL)	Equipment

Table 6 Instruments, software, and equipment required but not provided (continued)

Instrument, software, or equipment	Form
Racks for microcentrifuge tubes	Equipment
Two (2) 96-well cold racks	Equipment
Ice bucket	Equipment

Safety Precautions

1 The Agilent SARS-CoV-2 qRT-PCR Dx Kit workflow should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of patient samples and controls to prevent false positive results.

2 This test has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.

3 Carefully read the entire Instructions for Use.

4 Samples and controls should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with 2019-nCoV. https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html

5 Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples. If a spill occurs, immediately disinfect.

6 Specimens may be infectious. Use Universal Precautions when performing this assay. Proper handling and disposal methods should be established by the laboratory director. Only personnel adequately trained in handling infectious materials should be permitted to perform this diagnostic procedure.

7 If infection with 2019-nCoV is suspected based on current clinical screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.

8 Use only supplied or specified disposable laboratory ware.

9 Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.

10 PCR-based assays are sensitive to accidental introduction of amplification products from previous PCR reactions. Any contamination of the test samples or reagents can result in an incorrect result. Workflow in the laboratory must proceed in a unidirectional manner. Use the following best-practices to prevent PCR product contamination of samples throughout the workflow:

- Assign separate pre-PCR and post-PCR workstations and use dedicated supplies and reagents in each area. In particular, never use materials designated to post-PCR work for pre-PCR segments of the workflow. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.

- Maintain clean work areas. Clean pre-PCR surfaces daily and in between each assay using a 10% bleach solution and/or a product such as DNA*Zap* or RNase AWAY. Remove residual bleach with 70% ethanol.

- Wear a clean lab coat and clean, powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.

- Change aerosol barrier pipette tips between all manual liquid transfers.

- When extracting nucleic acid from samples, use proper aseptic technique to minimize the risk of cross-contamination between samples and avoid the inadvertent introduction of nucleases into the samples.

- When possible, keep reagent and reaction tubes capped or covered.

11 Do not eat, drink, smoke, or apply cosmetic products in the work areas.

12 Modifications to assay reagents, assay protocol, or instrumentation are not permitted.

13 Reagents must be stored and handled as specified in **Table 2** on page 11 and in **"Storage and Handling"** on page 17.

14 Do not use the kit after the indicated expiry date.

15 Dispose of waste in compliance with local, state, and federal regulations.

16 Safety Data Sheets are available on www.agilent.com.

17 Do not use material that may contain Guanidinium thiocyanate or any guanidine-containing materials on the real-time PCR instrument. Highly reactive and/or toxic compounds may form if combined with sodium hypochlorite (bleach).

18 Positive results are indicative of the presence of SARS-CoV-2 RNA.

Storage and Handling

Product packaging

Upon receipt of Agilent SARS-CoV-2 qRT-PCR Dx Kit, carefully inspect the product box for any visible signs of damage. If damage to the product box is detected, contact Agilent Technical Support.

Reagent storage, handling, and stability

- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on ice or a cold rack at all times during preparation and use.
- Controls must be thawed and kept on ice or a cold rack at all times during preparation and use.
- Refer to **Table 2** on page 11 for storage temperatures for the Agilent SARS-CoV-2 qRT-PCR Dx Kit reagents.

Specimen collection, handling, and storage

Inadequate or inappropriate specimen collection, storage, and transport are likely to yield false test results. Training in specimen collection is highly recommended due to the importance of specimen quality. CLSI MM13-A may be referenced as an appropriate resource.

- Collecting the Specimen
  - Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (2019-nCoV) https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
  - Follow specimen collection device manufacturer instructions for proper collection methods.
- Transporting Specimens
  - Clinical material collected from patient placed in an appropriate transport system. For the Agilent SARS-CoV-2 qRT-PCR Dx Kit, this includes NP specimens in viral transport medium (VTM), universal transport medium (UTM), saline, Liquid Amies, or specimen transport medium (STM).

3 Instructions for Extracting Nucleic Acids

This chapter contains instructions for extracting RNA from clinical test samples and the Human Specimen Control.

Extract Nucleic Acids

Performance of the Agilent SARS-CoV-2 qRT-PCR Dx Kit is dependent upon the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been qualified and validated for recovery and purity of RNA for use with the kit.

Manufacturer's recommended procedures (except as noted in recommendations below) are to be followed for sample extraction. Human Specimen Control must be included in each extraction batch.

QIAGEN QIAsymphony DSP Virus/Pathogen Midi Kit, automation protocol

Recommendation: Utilize 140 µl of sample and elute with 60 µl of buffer.

MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit, automation protocol

Recommendation: Utilize 200 µl of sample and elute with 50 µl of buffer.

4   Instructions for Preparing the qRT-PCR Reactions

This chapter contains instructions for preparing the quantitative reverse transcription PCR (qRT-PCR) reactions for test samples and control samples.

Agilent

Set Up the qRT-PCR Experiment on the Real-Time PCR System

Prior to setting up the qRT-PCR reaction plate, set up the experiment in the real-time PCR system so that the instrument is ready to run as soon as the reaction plate is prepared.

Follow the instructions for your specific real-time PCR system.

Agilent AriaMx/AriaDx Real-Time PCR System

- See **"Create and set up the AriaMx/AriaDx experiment (required if a template has not yet been created)"** on page 21 if you do not have a template file with the necessary settings.
- If you do have a template file to use, see **"Create the AriaMx/AriaDx experiment from the saved template"** on page 27.

> **NOTE** Power on the AriaMx or AriaDx instrument at least 3 hours prior to use. The instrument can be left on at all times to ensure it is always prepared for use.
>
> As per instrument specifications, the operating conditions are 20–30°C, 20–80% humidity, and ≤ 2000 meters elevation.

ABI 7500 Fast Real Time PCR Instrument

- See **"Create and set up the ABI 7500 Fast experiment (required if a template has not yet been created)"** on page 27 if you do not have a template file with the necessary settings.
- If you do have a template file to use, see **"Create the ABI 7500 Fast experiment from the saved template"** on page 32.

Bio-Rad CFX96 Touch Real-Time PCR Detection System

- See **"Create and set up the Bio-Rad CFX96 Touch Real-Time PCR experiment (required if saved protocol and plate files have not yet been created)"** on page 33 if you do not have saved protocol and plate files with the necessary settings.
- If you do have saved protocol and plate files, see **"Create the Bio-Rad CFX96 Touch Real-Time PCR experiment from saved protocol and plate files"** on page 38.

Create and set up the AriaMx/AriaDx experiment (required if a template has not yet been created)

If a template for the experiment already exists, go to **"Create the AriaMx/AriaDx experiment from the saved template"** on page 27.

Step 1. Create the experiment

1 From the instrument-connected PC, open the Aria software application to the Getting Started screen.

2 Under **New Experiment**, click **Experiment Types** (if not already selected).

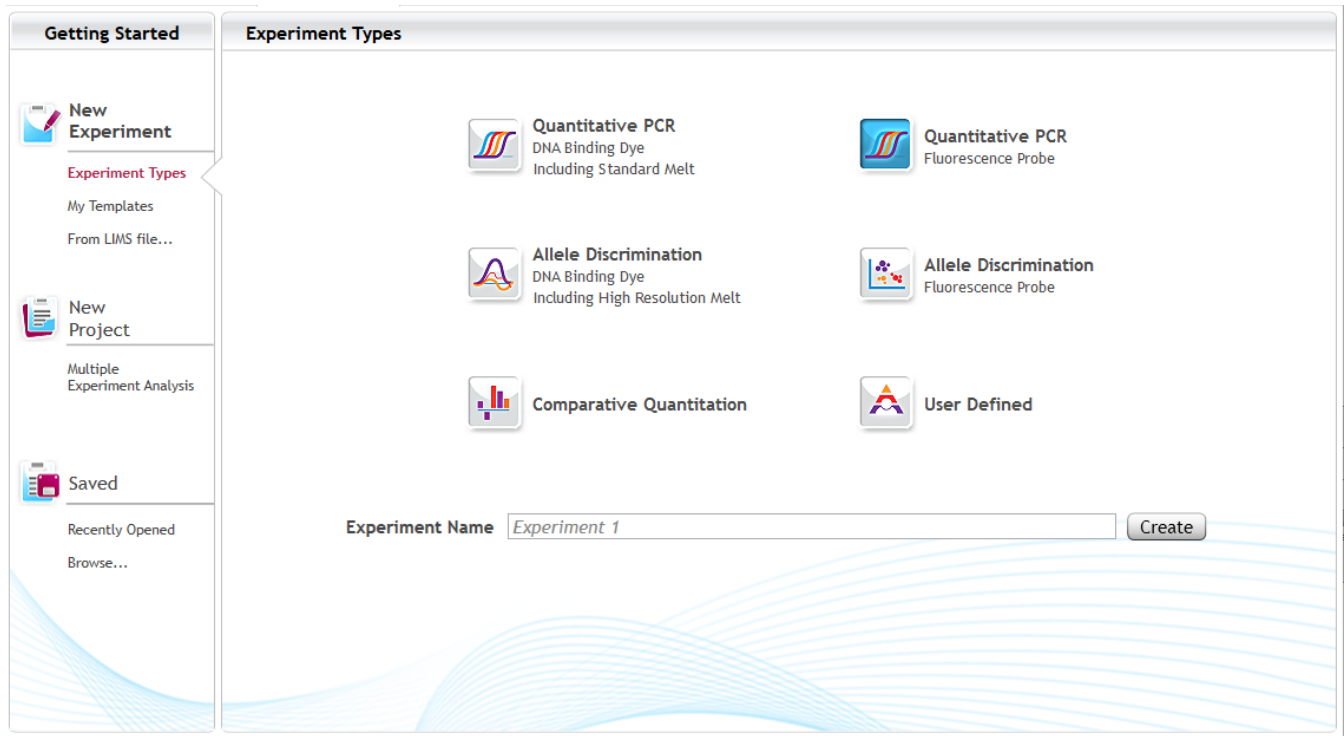**3** In the center of the screen, select **Quantitative PCR, Fluorescence Probe**, as shown in **Figure 2**.

Figure 2 Aria Getting Started screen - **Quantitative PCR, Fluorescence Probe** selected

4 Type a name for the experiment in the Experiment Name field and click **Create**.

The new experiment opens to the Plate Setup screen. All wells in the plate map are selected by default.

Selection of all 96 wells is appropriate if all wells of the plate will include a qRT-PCR reaction. If any of the plate wells will be empty, then de-select those wells at this step.

Step 2. Assign the well types and well names

5 In the Properties panel on the right side of the screen, expand the **Well type** drop-down list and select **Unknown**.

All wells are marked as Unknown in the plate map.

6 Assign well A12 as the No Template Control (NTC).

   a Click on well A12 on the plate map to select that individual well.

   b In the Properties panel on the right side of the screen, expand the **Well type** drop-down list and select **NTC**.

   Well A12 is marked as NTC in the plate map, while all other wells remain assigned to the Unknown well type.

7 On the plate map, reselect all wells.

Clicking the check box in the top left corner of the plate map selects all wells.

The plate setup now appears as shown in **Figure 3**.

Figure 3 Well type assignments on the Aria Plate Setup screen

8 Assign well names to the control wells and, if desired, to the wells used for test samples. Well names to be assigned to control wells are shown in **Table 7**. You can assign well names manually or by importing an Excel spreadsheet or comma-delimited text file that lists the well IDs alongside the well names to be assigned.

- To assign well names manually, change the Show setting from **Type** to **Name**. Select the well(s) on the plate, then type the name for the selected well(s) into the Well Name field.

- To assign well names from an Excel or text file, right-click on the plate map and select **Import Well Name**. In the dialog box that opens, select the Excel or text file. Consult the Aria help system for file formatting requirements.

Table 7 Well name assignments for control wells

Well ID	Well name
A12	NTC
B12	HSC*
H12	Pos

* If you have more than one Human Specimen Control sample that needs to be included on the plate, use additional wells in column 12 for those reactions and name the wells accordingly (e.g., HSC1, HSC2, etc.).

Step 3. Assign dyes and targets

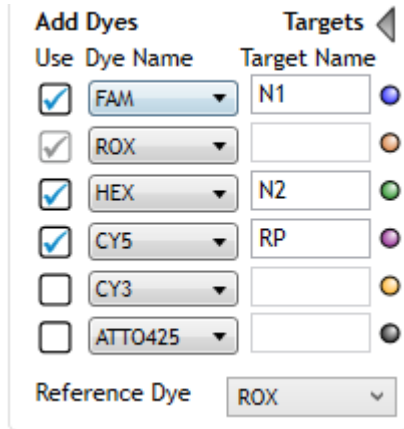**9** In the Properties panel, under **Add Dyes**, mark the check boxes for FAM, HEX, and Cy5.

Color-coded dots for each of the marked dyes appear in all wells of the plate map.

10 In the Reference Dye drop-down list, Select **ROX**.

A color-coded dot labeled "R" appears in all wells of the plate map.

11 Click the arrowhead ▷ next to **Targets**.

12 In the Target Name fields that appear, type target names according to **Figure 4**.

Figure 4   Dye and target assignments on the Aria Plate Setup screen

Step 4. Set up the thermal profile

13 On the left side of the screen, under **Set Up**, click **Thermal Profile**.

The Thermal Profile screen opens displaying the default thermal profile.

14 Add an RT segment to the start of the cycling program.

    a On the display, hover the cursor over the Hot Start segment. Click the + icon (as shown in **Figure 5**) that appears on the left side of the Hot Start segment.
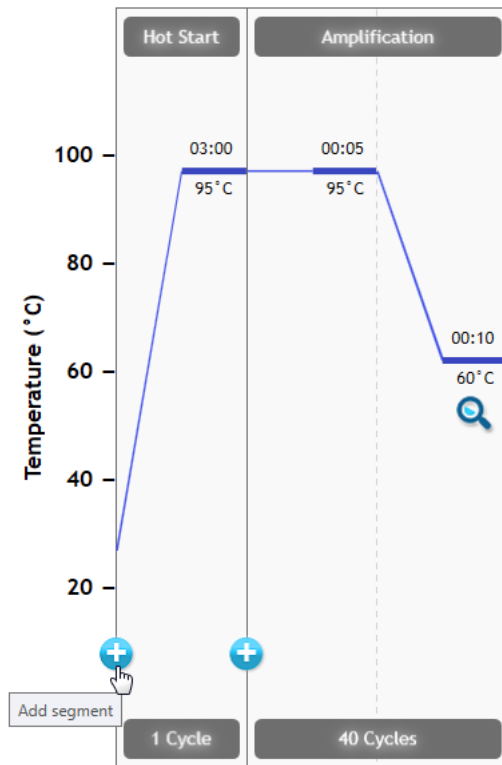
Figure 5   Adding a new segment on the Aria Thermal Profile screen

The program opens a placeholder for the new segment listing the available segment types.

b In the placeholder segment, click **RT**.

The thermal profile now appears as shown in **Figure 6**.
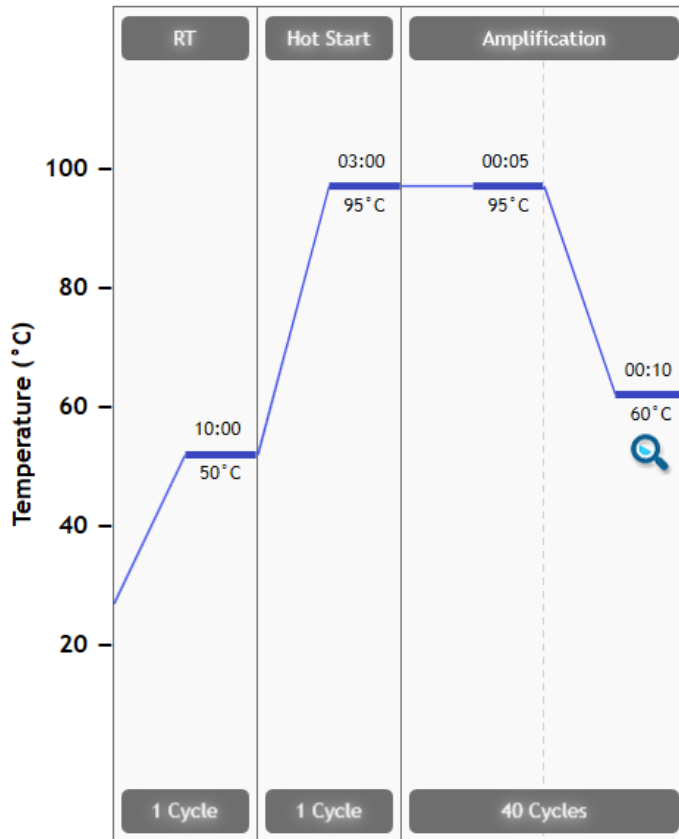
Figure 6   Thermal profile on the Aria Thermal Profile screen

15 Make sure that the thermal profile on your screen matches the thermal cycling program shown in **Table 8**.

Table 8   Thermal cycling program for AriaMx/AriaDx Real-Time PCR System

Segment Number	Number of Cycles	Duration	Temperature
1	1	10 minutes	50°C
2	1	3 minutes	95°C
3	40	5 seconds	95°C
		10 seconds	60°C

Step 5. Save the experiment as a template

16 Click **File > Save As Template**.

The Save As dialog box opens. The file type is set to **AriaMx Template Files** (file extension *amxt*) or **AriaDx Template Files** (file extension *adxt*).

17 Select a folder for the new template.

18 Into the File name field, type **Agilent SARS-CoV-2 qRT-PCR**.

19 Click **Save**.

The dialog box closes and the program saves the new template file to the designated folder.

For future assays, use the saved template to create and set up the qRT-PCR experiment as described in **"Create the AriaMx/AriaDx experiment from the saved template."**

At this point, proceed directly to **"Prepare the qRT-PCR Reactions"** on page 40.

Create the AriaMx/AriaDx experiment from the saved template

If a template of the experiment with the necessary plate setup and thermal profile has not been created, see **"Create and set up the AriaMx/AriaDx experiment (required if a template has not yet been created)"** on page 21.

1 From the instrument-connected PC, open the Aria software application to the Getting Started screen.

2 Under **New Experiment**, click **My Templates**.

3 In the Experiment Name field, type a name for the new experiment.

4 Select the **Agilent SARS-CoV-2 qRT-PCR** template and create the experiment.

- If the template is in the default folder, click directly on the template to select it and then click **Create** (or double- click directly on the template). The program creates the new experiment and opens the experiment to the Plate Setup screen.

- If the template is not in the currently selected folder, click the **Browse to Template** icon (shown below) to open the browser window. Browse to the folder containing the **Agilent SARS-CoV-2 qRT-PCR** template file. Select the file and click **Open**. The program creates the new experiment and opens the experiment to the Plate Setup screen.

At this point, proceed directly to **"Prepare the qRT-PCR Reactions"** on page 40.

Create and set up the ABI 7500 Fast experiment (required if a template has not yet been created)

If a template for the experiment already exists, go to **"Create the ABI 7500 Fast experiment from the saved template"** on page 32.

Step 1. Create the experiment

1 Turn on the ABI 7500 Fast instrument.

2 From the instrument-connected PC, open the 7500 System Software application.

3 On the Home screen, under **Set Up**, click **Advanced Setup**.

The Experiment screen opens.

4 In the **Experiment Menu** on the left side of the screen, under **Setup**, click **Experiment Properties** (if not already selected).

The settings for the Experiment Properties are displayed in the center of the screen.

5 Answer the questions on the screen using the selections and entries shown in **Table 9**

Table 9 Experiment Properties settings

Question	Selections/entries
How do you want to identify this experiment?	• Experiment Name: Type a unique name for the experiment • Barcode: Leave blank • User Name: Type your name • Comments: Type any desired comments or leave blank
Which instrument are you using to run the experiment?	7500 Fast (96 Wells)
What type of experiment do you want to set up?	Quantitation – Standard Curve
Which reagents do you want to use to detect the target sequence?	TaqMan® Reagents
Which ramp speed to you want to use in the instrument run?	Fast

Step 2. Define the targets and samples

6 In the **Experiment Menu** on the left side of the screen, under **Setup**, click **Plate Setup**. Make sure that the **Define Targets and Samples** tab is selected at the top.

The tools for defining the targets and samples are displayed in the center of the screen.

7 In the Define Targets table, create targets for N1, N2, and RP as shown in **Figure 7**. Click **Add New Target** to add a row to the table as needed.

For **Quencher** selection, select **NFQ-MGB** for all targets. For **Color** selection, use the default or select desired color.
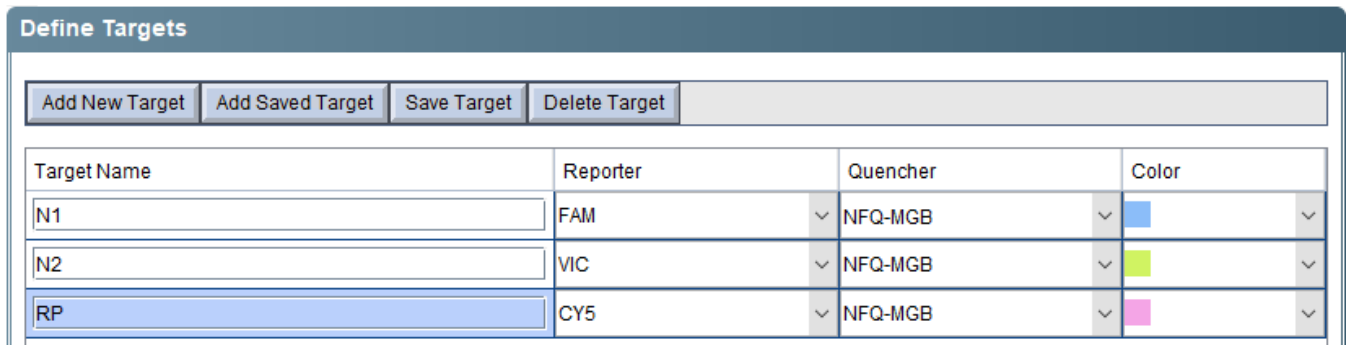
Figure 7 Target definitions on the 7500 Software Plate Setup screen

8 In the Define Samples table, create sample names as shown in **Figure 8**. Click **Add New Sample** to add a row to the table as needed.

The three samples are the Human Specimen Control (**HSC**), the No Template Control (**NTC**), and the positive control (**Pos**) with SARS-CoV-2 Synthetic Positive RNA Control. Note that the test samples are not assigned a sample name.

For **Color** selection, use the default or select desired color.

Figure 8   Sample definitions on the 7500 Software Plate Setup screen

Step 3. Assign targets and tasks

9 At the top of the screen, click the **Assign Targets and Samples** tab. Make sure that the **View Plate Layout** tab is selected.

The screen displays a plate map of the 96-well plate.

10 In the plate map, select all 96 wells.
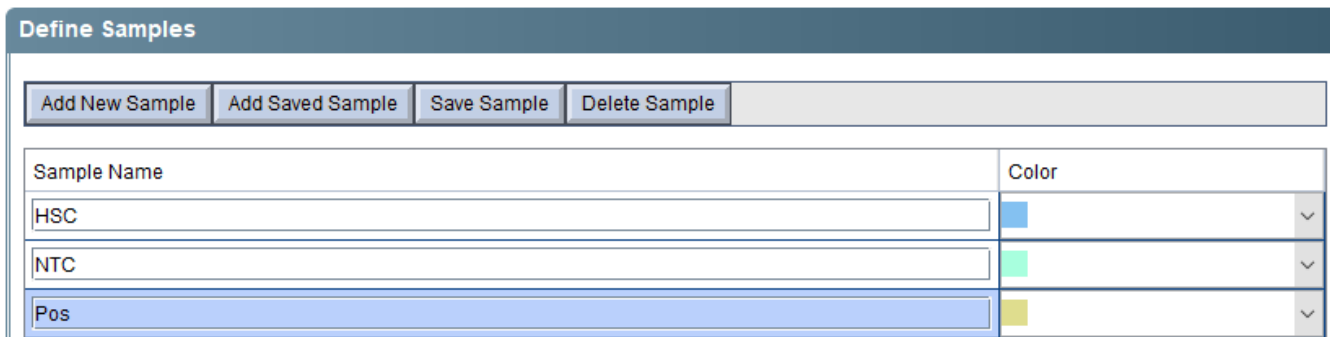
Selected wells are highlighted blue with a white oval in the center.

Selecting all 96 wells is appropriate if all wells of the plate will include a qRT-PCR reaction. If any of the plate wells will be empty, then do not select those wells at this step.

11 In the table under **Assign target(s) to the selected wells**, mark all three check boxes in the Assign column to indicate that all three targets will be monitored and reported in all wells. In the Task column, make sure "U" is selected for all three targets. Refer to **Figure 9**.

The plate map displays a symbol for each target in all 96 wells.

Figure 9   Target assignments on the 7500 Software View Plate Layout tab

12 Change the assigned task for the No Template Control well.

  a In the plate map, select well A12.

  b In the table under **Assign target(s) to the selected wells**, change the selection in the Task column to "N" for all three targets. Make sure that the check boxes in the Assign column remain marked.
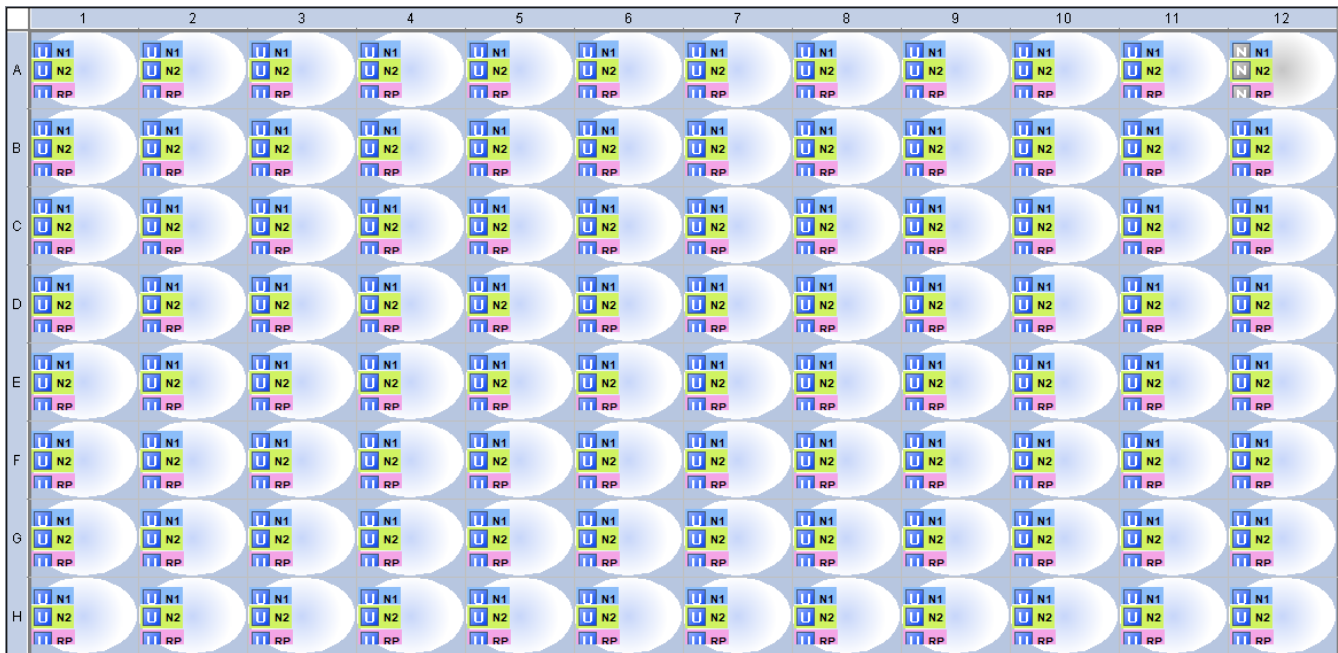
The plate map now appears as displayed in **Figure 10**.

Figure 10   Plate map on the 7500 System Plate Setup screen

Step 4. Assign control samples

13 Assign the No Template Control to well A12.

   a   Select well A12 on the plate map.

   b   In the table under **Assign sample(s) to the selected wells**, mark **NTC**.

   The sample name NTC is displayed in the selected well.

14 Assign the Human Specimen Control sample to well B12.

   a   Select well B12 on the plate map.

   If you have more than one Human Specimen Control sample that needs to be included on the plate, select the necessary number of additional wells in column 12 of the plate map.

   b   In the table under **Assign sample(s) to the selected wells**, mark **HSC**.

   The sample name HSC is displayed in the selected well(s).

15 Assign the SARS-CoV-2 Synthetic Positive RNA Control sample to well H12.

   a   Select well H12 on the plate map.

   b   In the table under **Assign sample(s) to the selected wells**, mark **Pos**.

   The sample name Pos is displayed in the selected well.

Step 5. Assign the reference dye

16 In the table under **Select the dye to use as the passive reference,** select **ROX**.

Step 6. Set up the run method

17 In the **Experiment Menu** on the left side of the screen, under **Setup**, click **Run Method**. Click the **Tabular View** tab at the top.

The tools for defining the reaction volume and thermal profile are displayed in the center of the screen.

18 Make sure that the **Reaction Volume Per Well** field is set to 20 µl. If it is not, then type **20** into the field.

19 Adjust the thermal profile to match that shown in **Figure 11**. The settings are also summarized in **Table 10**.

If you need to add a new holding stage to the beginning of the thermal profile for the 50°C reverse transcription step, follow steps below.

  a Select the left-most stage on the thermal profile image.

  b Click **Add Stage > Holding**.

  A new holding stage is added to the beginning of the thermal profile. Adjust temperature and duration to match those in **Figure 11**.
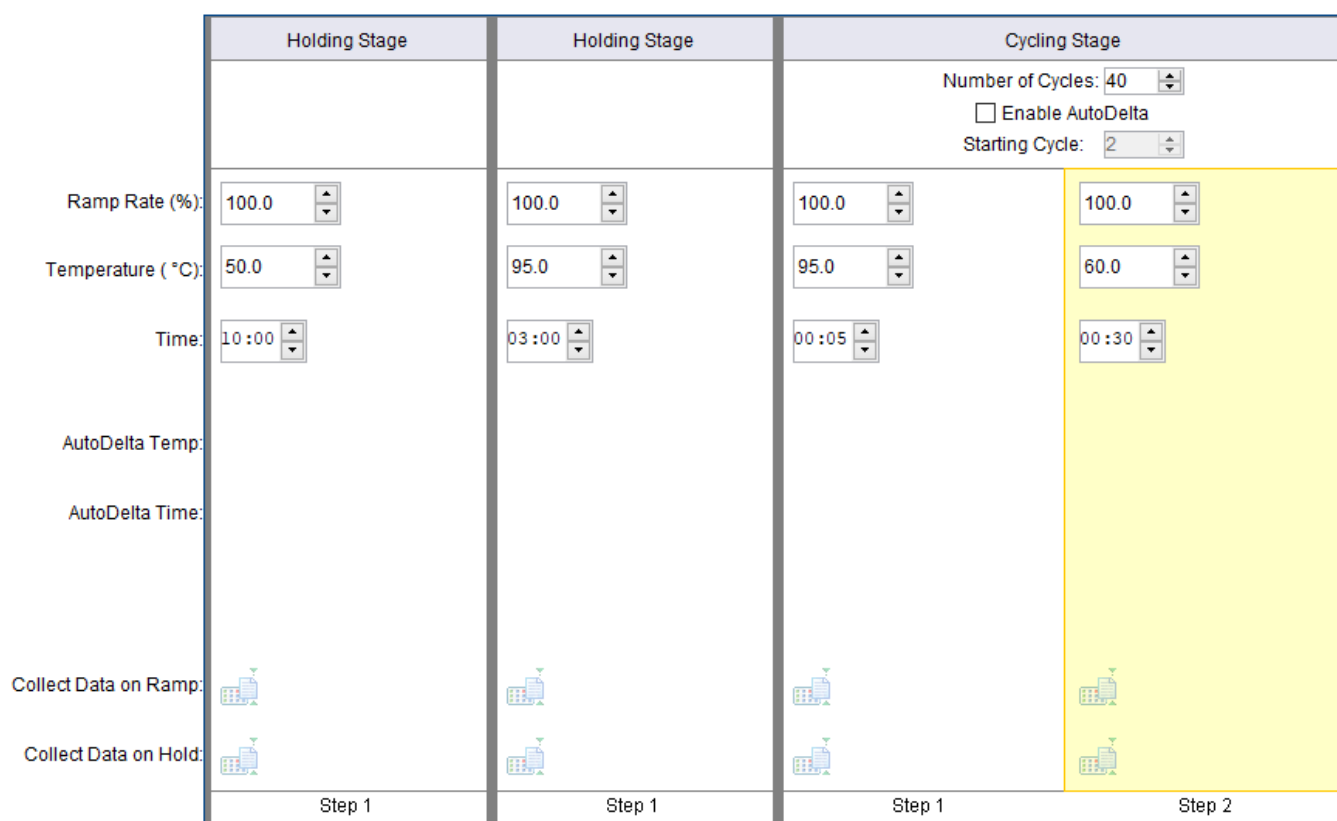

Figure 11　Thermal profile on the 7500 Software Run Method screen

Table 10 Thermal profile settings for 7500 Fast Real Time PCR Instrument

Segment Number	Number of Cycles	Duration	Temperature
1	1	10 minutes	50°C
2	1	3 minutes	95°C
3	40	5 seconds	95°C
		30 seconds	60°C

Step 7. Save the experiment

20 At the top of the screen, click the downward arrowhead next to the **Save** button to expand the menu of Save options.

21 Select **Save As** in the menu.

The Save As dialog box opens.

22 Select a folder for the new experiment file.

23 Into the File name field, type a name for the experiment.

24 Make sure that the file type is set to **Experiment Document Single files (*.eds)**.

25 Click **Save**.

The dialog box closes and the program saves the experiment file to the designated folder.

Step 8. Save the experiment as a template for future use

26 Click the downward arrowhead next to the **Save** button to expand the menu of Save options.

27 Select **Save As Template** in the menu.

The Save As Template dialog box opens.

28 Select a folder for the new template.

29 Into the File name field, type **Agilent SARS-CoV-2 qRT-PCR**.

30 Make sure that the file type is set to **Experiment Document Template files (*.edt)**.

31 Click **Save**.

The dialog box closes and the program saves the new template file to the designated folder.

For future assays, use the saved template to create and set up the qRT-PCR experiment as described in **"Create the ABI 7500 Fast experiment from the saved template."**

At this point, proceed directly to **"Prepare the qRT-PCR Reactions"** on page 40.

Create the ABI 7500 Fast experiment from the saved template

If a template of the experiment with the necessary plate setup and thermal profile has not been created, see **"Create and set up the ABI 7500 Fast experiment (required if a template has not yet been created)"** on page 27.

Step 1. Create the experiment

1 Turn on the ABI 7500 Fast instrument.

2 From the instrument-connected PC, open the 7500 System Software application.

3 On the Home screen, under **Set Up**, click **Template**.

The Open dialog box opens.

4 In the dialog box, navigate to the folder where the template is saved.

5 Double-click directly on the **Agilent SARS-CoV-2 qRT-PCR.edt** file.

The instrument initializes.

The 7500 System Software opens to the Experiment screen with the Plate Setup displayed.

Step 2. Save the experiment

6 At the top of the screen, click the downward arrowhead next to the **Save** button to expand the menu of Save options.

7 Select **Save As** in the menu.

The Save As dialog box opens.

8 Select a folder for the new experiment file.

9 Into the File name field, type a name for the experiment.

10 Make sure that the file type is set to **Experiment Document Single files (*.eds)**.

11 Click **Save**.

The dialog box closes and the program saves the experiment file to the designated folder.

At this point, proceed directly to **"Prepare the qRT-PCR Reactions"** on page 40.

Create and set up the Bio-Rad CFX96 Touch Real-Time PCR experiment (required if saved protocol and plate files have not yet been created)

If appropriate protocol and plate files already exist, go to **"Create the Bio-Rad CFX96 Touch Real-Time PCR experiment from saved protocol and plate files"** on page 38.

Step 1. Create the experiment

1 Turn on the CFX96 Touch Real-Time PCR instrument.

2 From the instrument-connected PC, open the Bio-Rad CFX Maestro software application.

3 In the Startup Wizard, set the **Select instrument** drop-down list to **CFX96** then click **User-defined**.

The Run Setup screen opens to the Protocol tab. The default thermal profile is displayed in the center of the screen.

Step 2. Set up the thermal profile

4 In the Express Load drop-down list, select **2-Step_Amp.prcl**.

The thermal profile updates to the default settings for a 2-step amplification protocol appropriate for use with fluorescent probes.
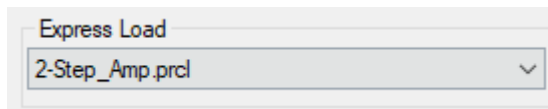

Figure 12 Express Load set to **2-Step_Amp.prcl** on the CFX Maestro Protocol tab

5 Click **Edit Selected**.

The Protocol Editor window opens.

6 In the Protocol Editor window, add a step at the start of the thermal profile for reverse transcription.

   a Select step 1 in the thermal profile image.

b In the Insert Step drop-down list, select **Before**.

c Click **Insert Step**.

A new step 1 is added before the selected step.

d Edit the settings for the new step to 50°C for 10 minutes.

7 Adjust the other steps of the thermal profile to match **Figure 13**. The settings are also summarized in **Table 11**. Make sure that the GOTO step (step 5) is set to repeat 39 times.
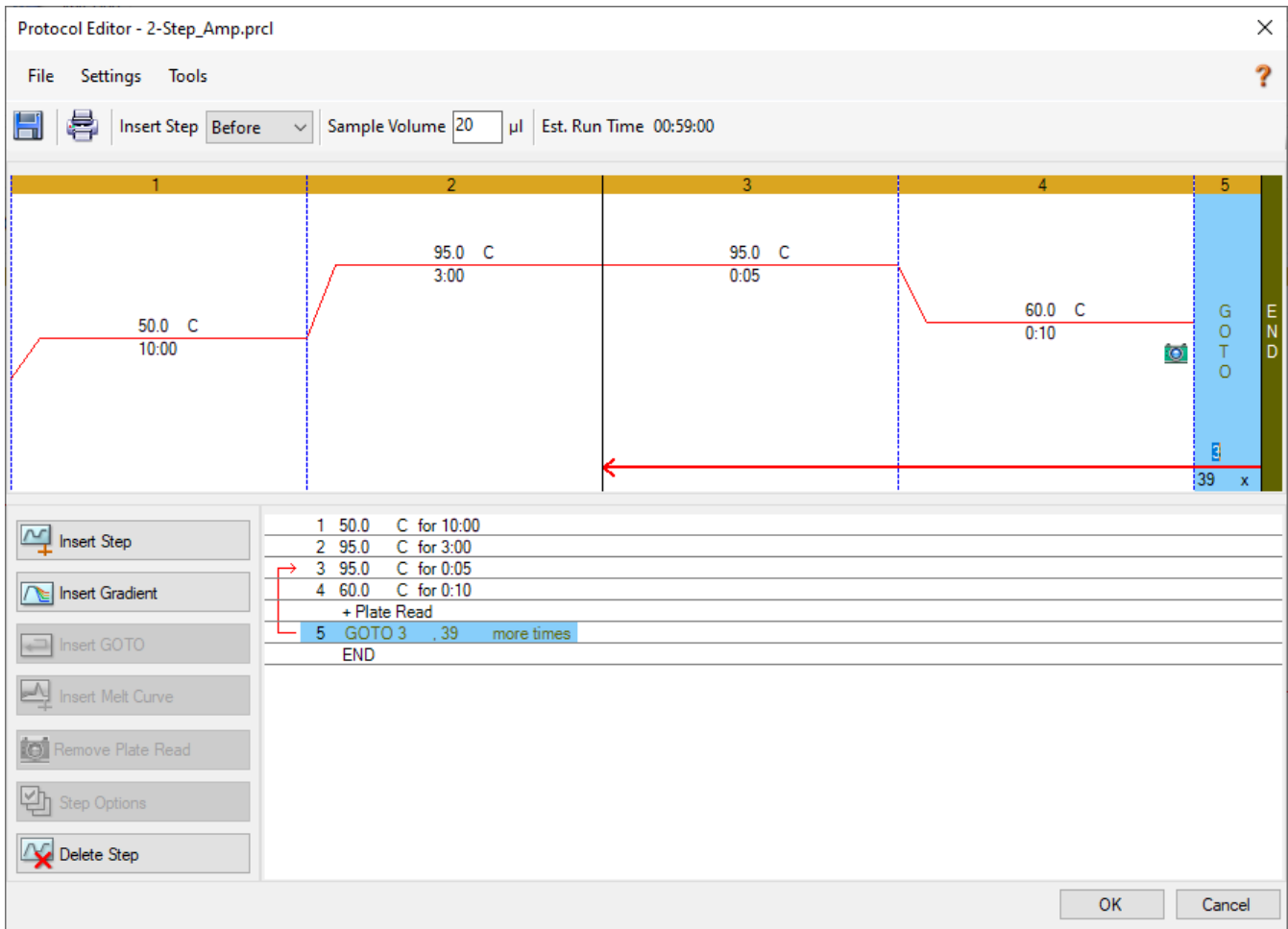

Figure 13   Thermal profile on the CFX Maestro Protocol Editor window

Table 11 Thermal cycling program for Bio-Rad CFX96 Touch Real-Time PCR Instrument

Segment Number	Number of Cycles	Duration	Temperature
1	1	10 minutes	50°C
2	1	3 minutes	95°C
3	40	5 seconds	95°C
		10 seconds	60°C

Step 3. Save the protocol file.

8 Click **Save**.

The Save As dialog box opens prompting you to save the protocol file for the experiment. The protocol file contains the settings from the Protocol tab of the Run Setup screen.

9 Select a folder for the protocol file.

10 Into the File name field, type **Agilent SARS-CoV-2 qRT-PCR Protocol**.

11 Make sure that the file type is set to **Protocol File (*.prcl)**.

12 Click **Save**.

The dialog box closes and the program saves the protocol file to the designated folder.

13 Click **OK** to close the Protocol Editor window.

For future assays, use the saved protocol file to set up the Protocol tab as described in **"Create the Bio-Rad CFX96 Touch Real-Time PCR experiment from saved protocol and plate files."**

Step 4. Select the fluorophores and assign target names

14 At the bottom of the Run Setup screen, click **Next**.

The Run Setup screen advances to the Plate tab. An image of the plate map is displayed in the center of the screen.

15 Make sure that the Scan Mode drop-down list is set to **All Channels**.

16 Click **Edit Selected**.

The Plate Editor window opens.

17 In the Plate Editor window, click **Select Fluorophores**.

The Select Fluorophores dialog box opens.

18 In the dialog box, mark the check boxes for fluorophores **FAM**, **HEX**, and **Cy5**. Clear the check boxes for all other fluorophores. Refer to **Figure 14**.
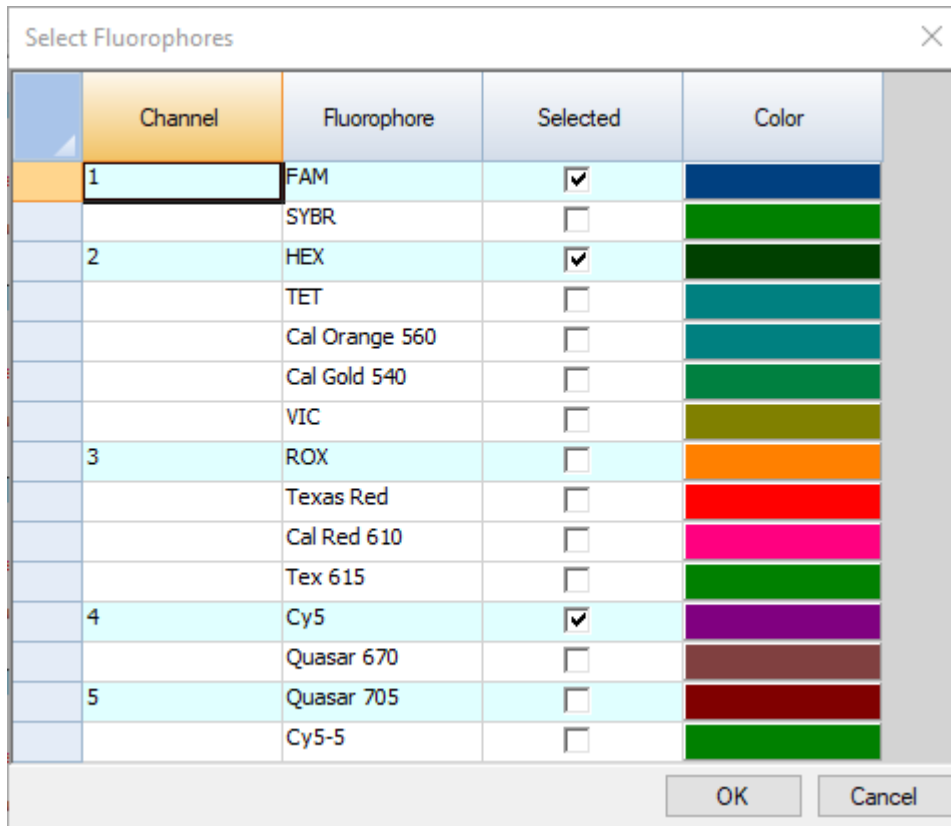
Figure 14 Fluorophores selections on the CFX Maestro Select Fluorophores dialog box

19 Click **OK**.

The Select Fluorophores dialog box closes. The three selected fluorophores are listed on the right side of the Plate Editor window under **Target Names**.

20 Select all wells on the plate map.

Selected wells are highlighted blue.

Selecting all 96 wells is appropriate if all wells of the plate will include a qRT-PCR reaction. If any of the plate wells will be empty, then do not select those wells at this step.

21 Under **Target Names** make sure that all three fluorophores are marked and that all wells display the names of all three fluorophores.

22 In the fields next to the fluorophore names, type target names for the fluorophores as shown in **Figure 15**. Press **Enter** after typing each name to apply the name to the plate wells.
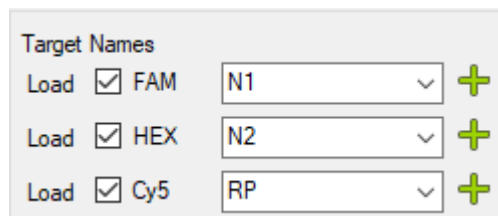

Figure 15 Target name assignments on the CFX Maestro Plate Editor window

Step 5. Assign sample types and sample names

23 Assign the No Template Control sample type.

    a Select well A12 on the plate map.

    b In the Sample Type drop-down list, select **NTC**.

       The **NTC** label is displayed at the top of well A12.

24 Make sure that the remaining wells are assigned to the Unknown sample type as indicated by the **Unk** label at the top of the wells, as shown in **Figure 16**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	NTC N1 N2 RP
B	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP
C	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP
D	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP
E	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP
F	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP
G	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP
H	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP	Unk N1 N2 RP

Figure 16   Sample types assignments on the CFX Maestro Plate Editor window

25 Assign sample name for the Human Specimen Control sample in well B12.

    a Select well B12 on the plate map.

       If you have more than one Human Specimen Control sample that needs to be included on the plate, select the necessary number of additional wells in column 12 of the plate map.

    b In the field under **Sample Names**, type **HSC**, as shown in **Figure 17**. Press **Enter**.

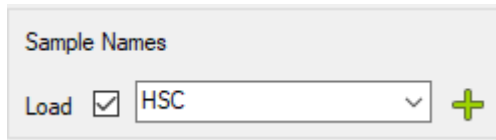   The sample name HSC is displayed in the selected well(s).

Figure 17 Addition of HSC sample name on the CFX Maestro Plate Editor window

26 Assign sample name for the SARS-CoV-2 Synthetic Positive RNA Control sample in well H12.

    a Select well H12 on the plate map.

    b In the field under **Sample Names**, type **Pos**. Press **Enter**.

       The sample name Pos is displayed in well H12.

Step 6. Save the plate file

27 Click **Save**.

The Save As dialog box opens prompting you to save the plate file for the experiment. The plate file contains the settings from the Plate tab of the Run Setup screen.

28 Select a folder for the plate file.

29 Into the File name field, type the name **Agilent SARS-CoV-2 qRT-PCR Plate**.

30 Make sure that the file type is set to **Plate File (*.pltd)**.

31 Click **Save**.

The dialog box closes and the program saves the plate file to the designated folder.

32 Click **OK** to close the Plate Editor window.

33 At the bottom of the Run Setup screen, click **Next**.

The Run Setup screen advances to the Start Run tab.

For future assays, use the saved plate file to set up the Plate tab as described in **"Create the Bio-Rad CFX96 Touch Real-Time PCR experiment from saved protocol and plate files."**

Create the Bio-Rad CFX96 Touch Real-Time PCR experiment from saved protocol and plate files

Step 1. Create the experiment

1 Turn on the CFX96 Touch Real-Time PCR instrument.

2 From the instrument-connected PC, open the Bio-Rad CFX Maestro software application.

3 In the Startup Wizard, set the **Select instrument** drop-down list to **CFX96** then click **User-defined**.

The Run Setup screen opens to the Protocol tab. The default thermal profile is displayed in the center of the screen.

Step 2. Load the protocol file

4 Click **Select Existing**.

The Select Protocol dialog box opens.

5 In the dialog box, navigate to the folder where the **Agilent SARS-CoV-2 qRT-PCR Protocol.prcl**. file is saved.

6 Double-click directly on the protocol file.

The settings from the protocol file are loaded into the experiment.

Step 3. Load the plate file

7 At the bottom of the Run Setup screen, click **Next**.

The Run Setup screen advances to the Plate tab. An image of the plate map is displayed in the center of the screen.

8 Click **Select Existing**.

The Select Plate dialog box opens.

9 In the dialog box, navigate to the folder where the **Agilent SARS-CoV-2 qRT-PCR Plate.pltd** file is saved.

10 Double-click directly on the plate file.

The settings from the plate file are loaded into the experiment.

11 At the bottom of the Run Setup screen, click **Next**.

The Run Setup screen advances to the Start Run tab.

At this point, proceed directly to **"Prepare the qRT-PCR Reactions"** on page 40.

Prepare the qRT-PCR Reactions

CAUTION Prepare the qRT-PCR plate immediately before use and keep it on ice or in a cold rack at all times until loaded into the real-time PCR instrument.

Step 1. Prepare the qRT-PCR reagent mixture and plate in the pre-PCR workstation

1 Thaw any frozen qRT-PCR reagents on ice. Refer to **Table 12** for a list of needed reagents. Keep the 10× SAR-CoV-2 Primer/Probe Mix Dx and Reference Dye Dx protected from light.

2 Prepare a 1:500 dilution of the provided Reference Dye Dx with nuclease-free water (for a final concentration of 30 nM in the reactions). Keep all solutions containing the reference dye protected from light.

   The diluted Reference Dye Dx, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

3 Prepare the reagent mixture by combining the components in **Table 12** in the order listed. Prepare a single reagent mixture for all samples (3 controls and up to 93 test samples) using multiples of each component listed below. Keep the reagent mixture on ice and protected from light. Prepare the reagent mixture just prior to use.

   • If preparing 96 reactions, prepare the reagent mixture in a 2-ml tube.

   • If preparing 48 reactions, prepare the reagent mixture in a 1.5-ml tube.

   For your convenience, **Table 12** includes volumes for the preparation of 1 reaction, 48 reactions, and 96 reactions.

Table 12 qRT-PCR Reagent Mixture

Component	Volume for 1 reaction	Volume for 48 reactions (including overage)	Volume for 96 reactions (including overage)
Nuclease-free water	1.5 µl	81 µl	162 µl
2× Brilliant III Ultra-Fast qRT-PCR Master Mix Dx	10 µl	540 µl	1080 µl
10× SAR-CoV-2 Primer/Probe Mix Dx	2 µl	108 µl	216 µl
100 mM DTT Dx	0.2 µl	10.8 µl	21.6 µl
Diluted Reference Dye Dx (from **step 2**)	0.3 µl	16.2 µl	32.4 µl
RT/RNase Block Dx	1 µl	54 µl	108 µl

4 Gently mix the reagent mixture on a vortex mixer without creating bubbles, then spin the tube in a microcentrifuge for 5 seconds.

5 Distribute 15 µl of reagent mixture to the wells of the 96-well plate using the following procedure.

   a Put a clean 8× tube strip in a 96-well cold rack.

   b Dispense 190 µl of reagent mixture (if running 96 reactions) or 100 µl of reagent mixture (if running 48 reactions) to each of the 8 tubes in the strip. If necessary, dislodge any large bubbles that may be present at the bottom of the tubes.

c With a multi-channel pipettor, transfer 15 μl of reagent mixture from the 8-well strip tube to the columns of the 96-well plate. Keep the plate on ice or in a 96-well cold rack, and protected from light, throughout the process.

> **NOTE** If you are only running 48 reactions, then leave columns 1 through 6 empty.

6 With the plate covered, take the plate to the Pre-PCR workstation area designated for sample addition. Keep the plate on ice or in a cold rack.

Step 2. Add the control and test samples to the qRT-PCR plate in the Pre-PCR workstation (sample addition area)

7 Dilute the SARS-CoV-2 Synthetic Positive RNA Control to a working stock of 10 copies/μl.

a Add 99 μl of nuclease-free water to a clean 1.5-ml tube. To this tube, add 1 μl of undiluted SARS-CoV-2 Synthetic Positive RNA Control. Mix well on a vortex mixer, then briefly spin in a microcentrifuge.

b Add 99 μl of nuclease-free water to another clean 1.5-ml tube. To this tube, add 1 μl of the diluted SARS-CoV-2 Synthetic Positive RNA Control prepared in **step a**. Mix well on a vortex mixer, then briefly spin in a microcentrifuge.

This tube contains the working stock of SARS-CoV-2 Synthetic Positive RNA Control, with a concentration of 10 copies/μl.

The working stock, if stored at 4°C, can be used within the day for setting up additional assays. Return the original stock of SARS-CoV-2 Synthetic Positive RNA Control to −80°C.

8 Set up the control reactions as shown in **Figure 18**.

- For the No Template Control (NTC), add 5 μl of nuclease-free water to well A12.
- For the Human Specimen Control (HSC), briefly vortex the RNA prepared from the Human Specimen Control. Then, add 5 μl of the RNA to well B12.

  If you have more than one HSC sample that needs to be included on the plate, use additional wells in column 12 as designated when setting up the plate in the qRT-PCR instrument software.

- For the SARS-CoV-2 Synthetic Positive RNA Control (Pos), add 5 μl of the diluted SARS-CoV-2 Synthetic Positive RNA Control to well H12.

9 Set up the reactions for the test samples (S1 through S93) as shown in **Figure 18**. Change gloves frequently to avoid contamination.

- Briefly vortex the RNA samples prepared from the test samples. Then spin in a microcentrifuge for 5 seconds.
- For each sample, add 5 μl to one well of the qRT-PCR plate. Carefully track which sample was added to each well. Change tips after each addition.
- If the qRT-PCR plate is to be sealed with an adhesive seal, then mix the reactions before sealing by briefly pipetting the mixtures up and down without creating bubbles.

> **NOTE** If you are only running 48 reactions, then leave the plate wells labeled for S1 through S48 empty.

10 Seal the plate with either an adhesive seal or tube cap strips.

11 If the plate is sealed with tube cap strips, briefly mix on a vortex mixer. (Do not use a vortex mixer for plates sealed with adhesive seal.)

12 Briefly spin the plate in a plate centrifuge.

13 If you are using the Agilent AriaMx/AriaDx Real-Time PCR System and sealed the plate with adhesive seal, then add a compression pad over the plate.

14 Proceed directly to performing qRT-PCR on your real-time PCR system.

- See **"Perform qRT-PCR on Agilent AriaMx/AriaDx Real-Time PCR System"** on page 44
- See **"Perform qRT-PCR on ABI 7500 Fast Real Time PCR Instrument"** on page 52
- See **"Perform qRT-PCR on Bio-Rad CFX96 Touch Real-Time PCR Detection System"** on page 58

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	NTC
B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	HSC
C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S89
D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S90
E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	S91
F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86	S92
G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87	S93
H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88	Pos

Figure 18   Plate setup for test samples 1–93 and control samples (No Template Control [NTC], Human Specimen Control [HSC], and Synthetic Positive RNA Control Dx [Pos])

5 Instructions for Performing qRT-PCR

This chapter contains instructions on performing the qRT-PCR cycling program on the selected Real Time PCR instrument.

Perform qRT-PCR on Agilent AriaMx/AriaDx Real-Time PCR System

This section describes the procedure for performing qRT-PCR on an Agilent AriaMx or AriaDx Real-Time PCR System.

Run the qRT-PCR program on the AriaMx/AriaDx system

1 Make sure that the AriaMx/AriaDx instrument is powered on.

2 From the instrument-connected PC, open the experiment that you created earlier in the Aria application.

3 Navigate to the Thermal Profile screen, Run Status screen, or Raw Data Plots screen.

4 Click **Run**.

The Instrument Explorer dialog box opens.

5 Locate the instrument that you will be using for the run and click **Send Config**. (See the AriaMx or AriaDx Set Up and User Guide for instructions on searching for and adding instruments.)

   • If this is the first time you have connected to an instrument since last launching the Aria program, the Login dialog box opens. Select your Username from the drop- down list, type your login password into the Password field, and click **Login**. To log in with a different user account, right- click on the instrument name and click **Log off current user**. You can then log in using the desired user account.

   • If the experiment is not yet saved, you will be prompted to save the experiment before proceeding.

6 Take your reaction plate over to the instrument and load it into the thermal block.

7 On the instrument touchscreen, open the primed experiment to the Thermal Profile screen and press **Run Experiment**.

The instrument starts running the experiment.

8 Return to the PC program. The program directs you to the Run Status screen, where you can monitor the progress of the run.

Monitoring the run is not required. If you close the Aria software prior to completion of the run, make note of where the post-run experiment file will be saved.

Assign data analysis settings for the AriaMx/AriaDx experiment

Step 1. View the amplification plots for all targets in all wells

1 When the run is complete, open the experiment file in the Aria software.

2 Click **Analysis Criteria** on the left side of the screen.

The Analysis Criteria screen opens, with settings for specifying the settings for analysis.

3 Make sure that all wells, well types, and targets are selected for analysis, as shown in **Figure 19**.

> **NOTE** If the plate contains empty wells, make sure those are assigned to the Blank well type on the Plate Setup tab.
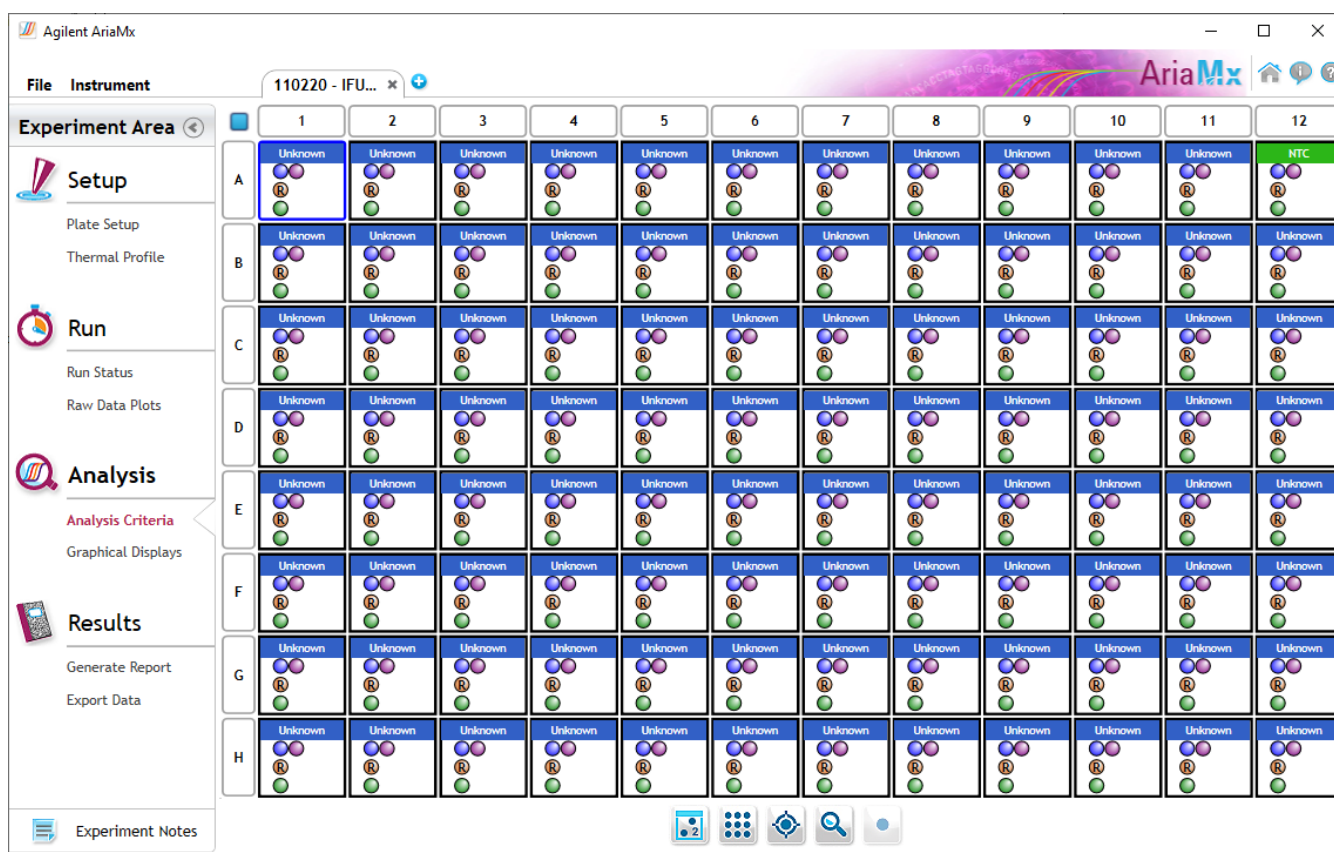
Figure 19 Aria Analysis Criteria screen

4 Click **Graphical Displays** on the left side of the screen.

The Graphical Displays screen opens, which displays the data with tools for setting analysis parameters.

5 Make sure that the screen is displaying the Amplification Plots graph and that the default analysis parameters match those shown in **Figure 20**.

If you do not see the full panel of analysis parameters shown in **Figure 20**, then click the downward arrowhead just under the **Smoothing** setting to expand the panel.

Note that the default Threshold Fluorescence values in your experiment will likely differ from those shown in **Figure 20**. The software calculates the default values based on the level of fluorescence noise in the background cycle range. Consequently, the default values will vary between experiments.
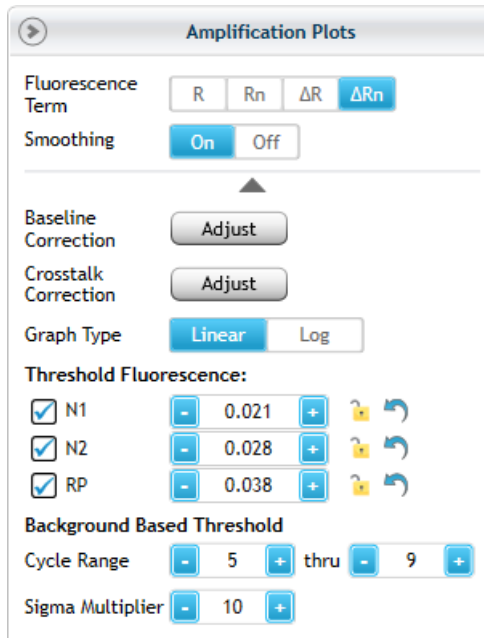
Figure 20 Default settings for Amplification Plots in the Aria software (Threshold Fluorescence values will vary between experiments)

6 Under **Background Based Threshold**, make sure that the default cycle range is 5 through 9.

7 Check for amplification plots with early amplification (i.e., prior to cycle 9). Exclude any test samples with early amplification from the analysis and re-test at a lower concentration.

In test samples with unusually high concentrations of viral RNA, amplification of the viral targets may reach detectable levels at a very early cycle number (<9). As part of the baseline correction calculations, the software may interpret such early amplification signals as background noise and fail to assign a Cq value to the viral targets in those samples.

Monitor for the presence of such test samples by viewing the Amplification Plots without baseline correction (Fluorescence Term R or Rn). If any of the test samples do show signs of amplification prior to cycle 9, then remove those wells from the analysis by de-selecting them on the Analysis Criteria screen. Re-run the qRT-PCR reaction for the sample by diluting the nucleic acid 1:100 using the appropriate elution buffer.

Step 2. Assess threshold values

8 In the Graph Type setting, switch the selection from **Linear** to **Log**. Make sure that the Fluorescence Term is set to Δ**Rn**.

Viewing the Amplification Plots in log values provides a better view of the background signal noise. An example is shown in **Figure 21**. The threshold line for each target is displayed as a horizontal line.
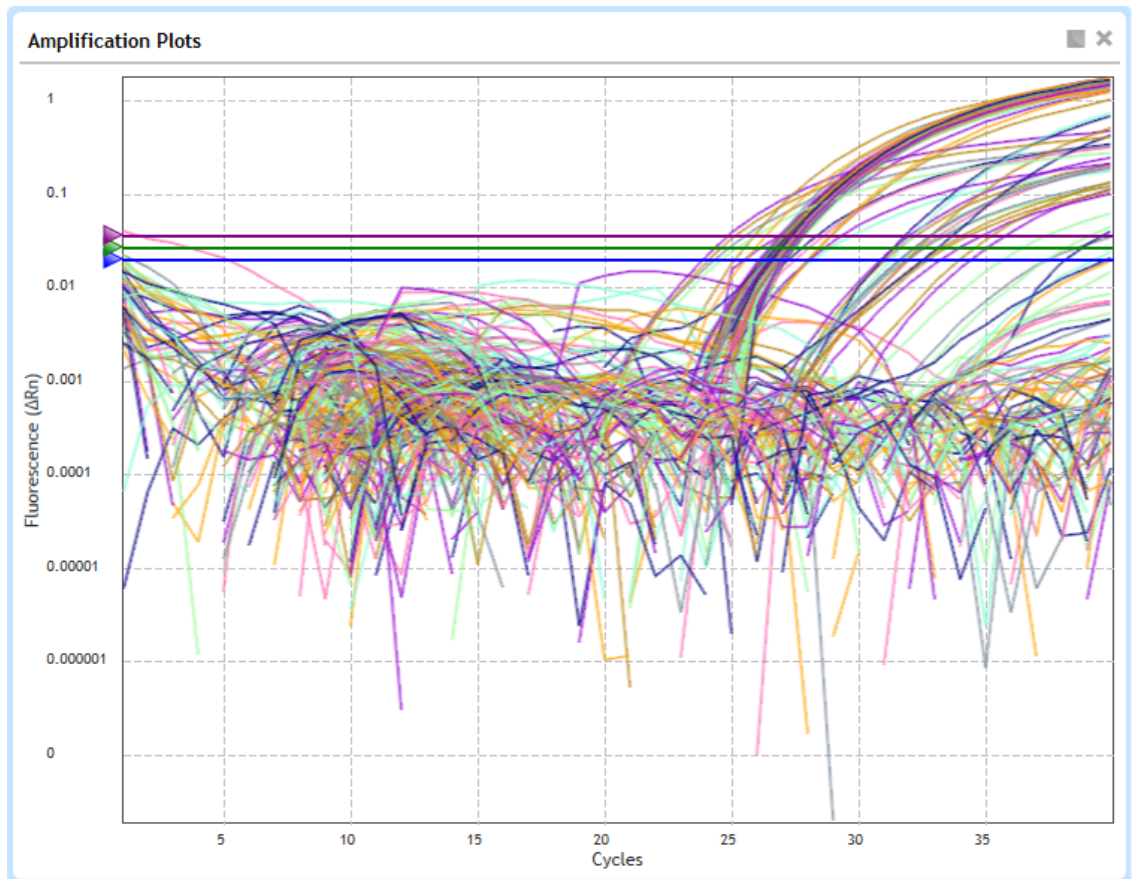
Figure 21 Aria Amplification Plots in log scale – target thresholds displayed as horizontal lines

9　Visually assess the amplification plots and default threshold for the N1 target.

　a　Click the display targets icon located below the graph. ⊙

　b　In the menu that opens, clear the boxes for all other targets so that only the N1 target is displayed in the Amplification Plots graph.

　c　Determine if the threshold is both high enough to be above the background noise and low enough to include plots in the exponential amplification phase (refer to **Figure 23**). Based on this determination, proceed as directed in **Table 13**.

10　Repeat **step 9** for the N2 target and again for the RP target.

11　Under **Threshold Fluorescence**, make sure that all three targets are marked and that all three Threshold Fluorescence values are locked, as shown in **Figure 22**.

12　Click the display targets icon located below the graph. ⊙ Make sure all targets are selected.

Threshold Fluorescence:

☑ N1	0.021	🔓 ↺
☑ N2	0.028	🔓 ↺
☑ RP	0.038	🔓 ↺

Figure 22 Targets selected and Threshold Fluorescence values locked
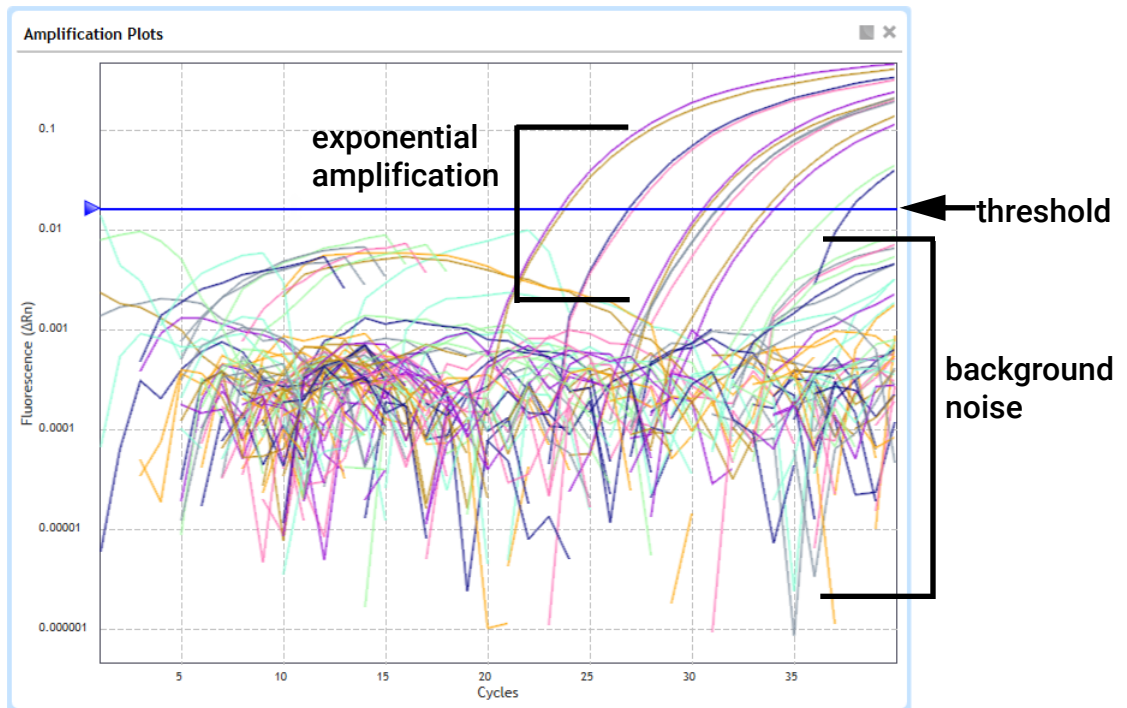
Figure 23   Aria Amplification Plots for N1 target

Table 13 Verifying optimal threshold setting in the Aria software

Threshold position	Description	Action
Optimal position	Above background noise and includes all plots in exponential amplification phase	• Lock the Threshold Fluorescence value on the right side of the screen by clicking the lock icon. When the value is locked, the lock icon is in the closed position. Locking the value prevents the value from changing if any of the analysis criteria are changed.

Table 13 Verifying optimal threshold setting in the Aria software

Threshold position	Description	Action
Too high	Some plots in exponential amplification phase do not pass threshold	• Look for wells with exceptionally high background noise. Such outlier wells can cause the software to set the threshold too high. • Go back to the Analysis Criteria screen to exclude that well from the analysis. Software will recalculate the threshold once the outlier well is excluded. Alternatively, use the mouse to manually drag the threshold line on the graph to a new position. • Verify new threshold is in an optimal position and lock the Threshold Fluorescence value as described above. • Once the new value is locked, go back to the Analysis Criteria screen once again to re-include the outlier well in the analysis.* * If the amplification plot for the outlier well does not show exponential amplification at any cycle number, then make sure that the lowered threshold does not cause the software to assign a Cq value to the well (due to background signal noise crossing the threshold). If it does, then try modifying other analysis settings for that well, e.g., adjusting the Baseline Correction setting.
Too low	Some plots are not above the background noise.	• Increase the threshold to just above the level of background noise.

Step 3. Verify the results in the NTC well

13 In the Results Table, locate the No Template Control (NTC) reaction, located in well A12.

14 Make sure that the Cq column for all three targets reads "No Cq" or has a Cq value > 37.00.

If the NTC reaction has a Cq value ≤ 37.00 for any of the targets, then sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

Export the data from the Aria software

Step 1. Define and save export settings

If the export settings have already been saved, go directly to **"Step 2. Export the data"** on page 51.

1 On the left side of the screen, under **Results**, click **Export Data**.

The Export Data screen opens.

2 In the Export Configuration panel, select a file type for the exported data, e.g., **Excel**.

3 Under **Items**, make sure that the Tabular Results check box is marked. Clear the check boxes for the other items. Refer to **Figure 24**.
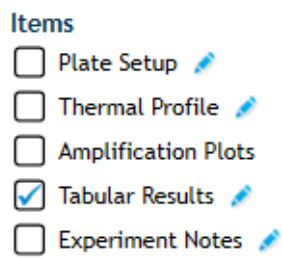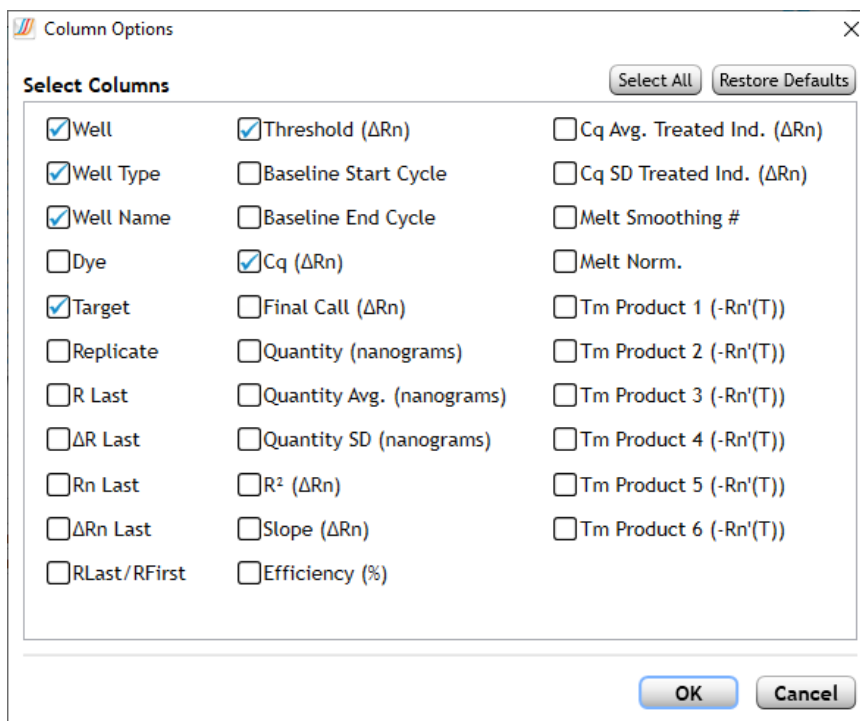
Figure 24   Aria software Export Data screen – Items to export

4 Click the pencil icon next to **Tabular Results** to customize which data columns to include.

The Column Options dialog box opens.

5 Adjust the settings so that the only columns marked for inclusion are those shown in **Figure 25**.

Figure 25   Aria Column Options dialog box

6 Click **OK** to save your changes and close the dialog box.

7 Save the export settings as a new definition.

    a In the Export Configuration panel, expand the Definition drop-down list.

    b Click **Add New**.

       The Add New Definition dialog box opens.

    c In the Definition Name field, type **Agilent SARS-CoV-2 qRT-PCR Data**. Refer to **Figure 26**.

    d Make sure the Use Current Settings check box is marked.

    e Click **Add**.
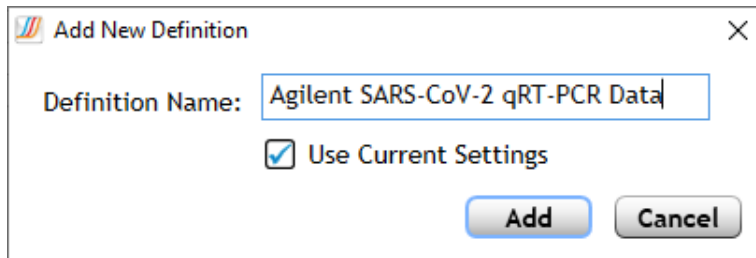
Figure 26   Aria Add New Definition dialog box

Step 2. Export the data

8 In the Export Configuration panel, make sure that the Definition drop-down list is set to **Agilent SARS-CoV-2 qRT-PCR Data**.

9 Click **Export Data**.

The report opens in the default application for the selected file type.

10 Review the Cq data for each of the test samples and controls to interpret the results. See **Chapter 6**, "Analysis and Results."

Perform qRT-PCR on ABI 7500 Fast Real Time PCR Instrument

This section describes the procedure for performing qRT-PCR on an Applied Biosystems 7500 Fast Real Time PCR Instrument.

Run the qRT-PCR program on the ABI 7500 Fast Real Time PCR Instrument

1   Make sure that the ABI 7500 Fast instrument is powered on.

2   From the instrument-connected PC, open the experiment that you created earlier in the 7500 software.

3   On the instrument, push the tray door to open the lid. Load your reaction plate into the instrument's thermal block.

4   Push the tray door to close the lid.

5   On the PC, click **START RUN**.

The instrument starts running the experiment.

Assign data analysis settings for the ABI 7500 Fast experiment

Step 1. View the amplification plots for all targets in all wells

1   When the run is complete, open the experiment in the Design & Analysis software application.

2   At the top of the screen, click the Quality Check tab, if not already selected. In the drop-down list, make sure **Amplification Plot** is selected.

The screen displays the amplification plots, a table of the results in each well, and a diagram of the plate map.

> **NOTE**   If the plate contains empty wells, omit all targets for those wells from the analysis and reanalyze.

3   Click **Actions > Primary Analysis Setting**.

The Primary Analysis Settings dialog box opens. The default settings in this dialog box are shown in **Figure 27**.

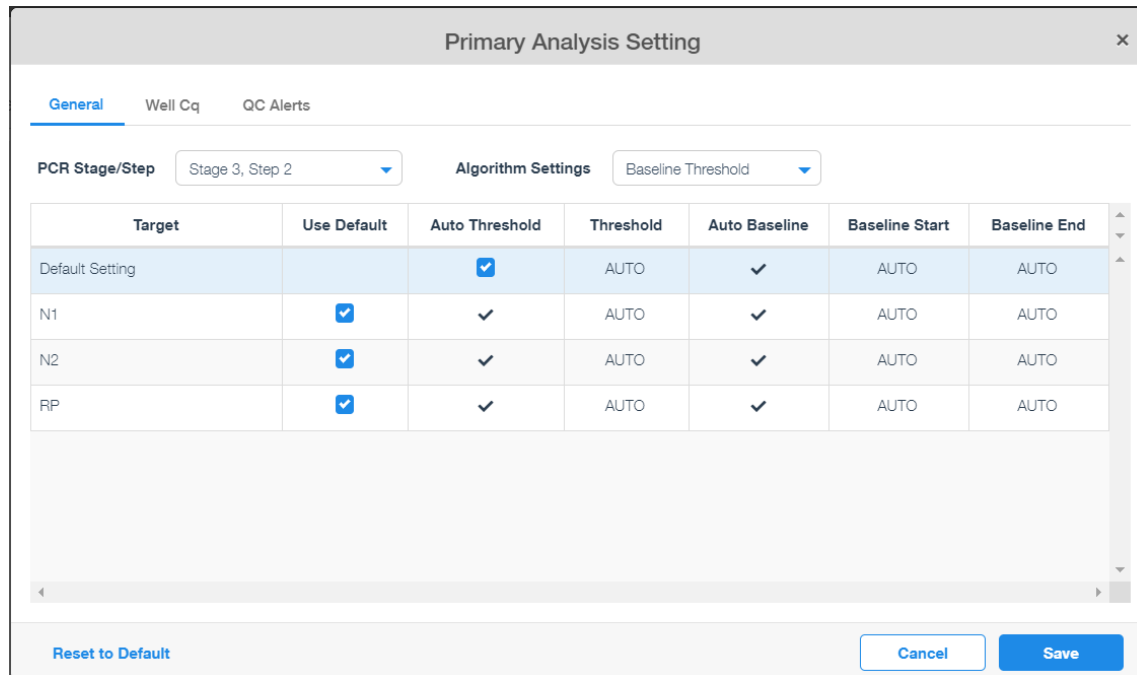4   Make sure that the setting match those shown in **Figure 27**. If necessary, click **Reset to Default**.

Figure 27 Default settings in the Design & Analysis Primary Analysis Setting dialog box

5 Click **Save**.

The Primary Analysis Setting dialog box closes.

6 Make sure that the Analyze button has a green check mark in the corner. If not click **Analyze**.

7 Check for amplification plots with early amplification (i.e., prior to cycle 9). Omit any test samples with early amplification from the analysis and re-test at a lower concentration.

In test samples with unusually high concentrations of viral RNA, amplification of the viral targets may reach detectable levels at a very early cycle number (<9). As part of the baseline correction calculations, the software may interpret such early amplification signals as background noise and fail to assign a Cq value to the viral targets in those samples.

Monitor for the presence of such test samples by viewing the Amplification Plot without baseline correction (set **Y Value** to **Rn**). If any of the test samples do show signs of amplification prior to cycle 9, then remove those wells from the analysis by omitting them on the Quality Check screen. Re-run the qRT-PCR reaction for the sample by diluting the nucleic acid 1:100 using the appropriate elution buffer.

Step 2. Assess threshold values

8 Click the Settings icon just above the Amplification Plot graph

The Settings dialog box opens to the General tab.

9 Make sure that the Y Value setting is set to Δ**Rn**.

10 In the Y Scale drop-down list, switch the selection from **Linear** to **Log**, as shown in **Figure 28**.

Viewing the Amplification Plots in log values provides a better view of the background signal noise. An example is shown in **Figure 29**. The threshold line for each target is displayed as a horizontal line.

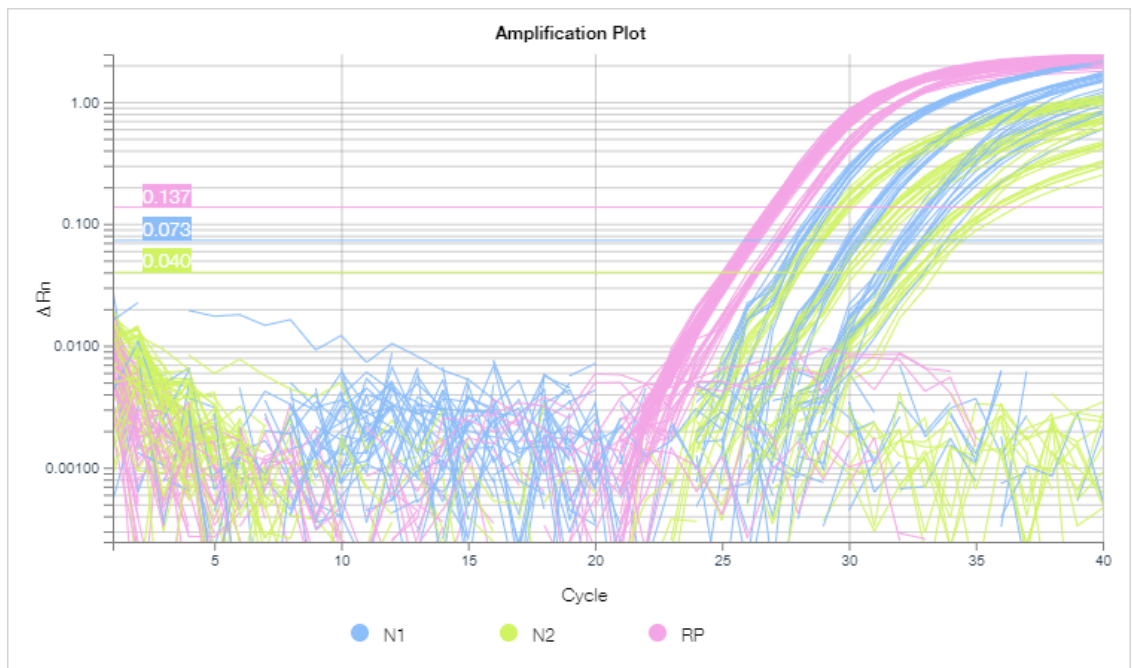**Figure 28** Design & Analysis General Settings for Amplification Plot

Figure 29 Design & Analysis Amplification Plots in log scale – target thresholds displayed as horizontal lines

11 Visually assess the amplification plots and default threshold for the N1 target.

    a On the left side of the screen, expand the Targets drop-down list.

    b Select only the N1 target, then collapse the drop-down list.

    c Determine if the threshold is both high enough to be above the background noise and low enough to include plots in the exponential amplification phase (refer to **Figure 30**). Based on this determination, proceed as directed in **Table 14**.

12 Repeat **step 11** for the N2 target and again for the RP target.

13 On the left side of the screen, under **Targets**, click **Clear all** to remove filtering by target.
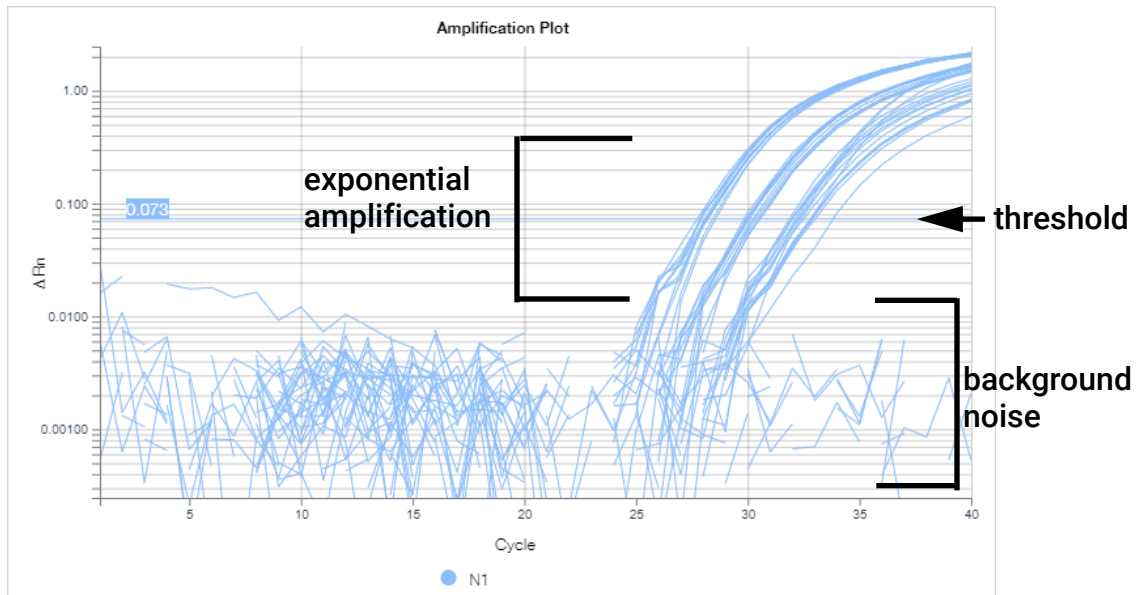
Figure 30   Design & Analysis Amplification Plots for N1 target

Table 14 Verifying optimal threshold setting in the Design & Analysis software

Threshold position	Description	Action
Optimal position	Above background noise and includes all plots in exponential amplification phase	• Leave the thresholds at their current values.
Too high	Some plots in exponential amplification phase do not pass threshold	• Look for wells with exceptionally high background noise. Such outlier wells can cause the software to set the threshold too high. • Select the outlier well on the plate map. • In the toolbar directly above the plate map, click the button with three dots. In the menu that opens, click **Omit Wells**. • Click **Analyze**. Software will recalculate the threshold once the outlier well is excluded. Alternatively, use the mouse to manually drag the threshold line on the graph to a new position. • Select all wells on the plate map to view all amplification plots. Verify new threshold is in an optimal position. • Re-click the button with three dots. In the menu that opens, click **Unomit Wells** to re-include the outlier well in the analysis.* * If the amplification plot for the outlier well does not show exponential amplification at any cycle number, then make sure that the lowered threshold does not cause the software to assign a Cq value to the well (due to background signal noise crossing the threshold). If it does, then try modifying other analysis settings for that well, e.g., adjusting the Baseline Start and/or Baseline End cycle numbers.
Too low	Some plots are not above the background noise.	• Increase the threshold to just above the level of background noise.

Step 3. Verify the results in the NTC well

14 In the Well Table, locate the No Template Control (NTC) reaction, located in well A12.

15 Make sure that the Cq column for all three targets reads "Undetermined" or has a Cq value > 37.00.

If the NTC reaction has a Cq value ≤ 37.00 for any of the targets, then sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

Export the Well Table data from the Design & Analysis software to a CSV file

Step 1. Customize the columns included in the Well Table

1 In the toolbar immediately above the Well Table, click **View**.

A menu opens, as shown in **Figure 31**, listing all of the available table columns.
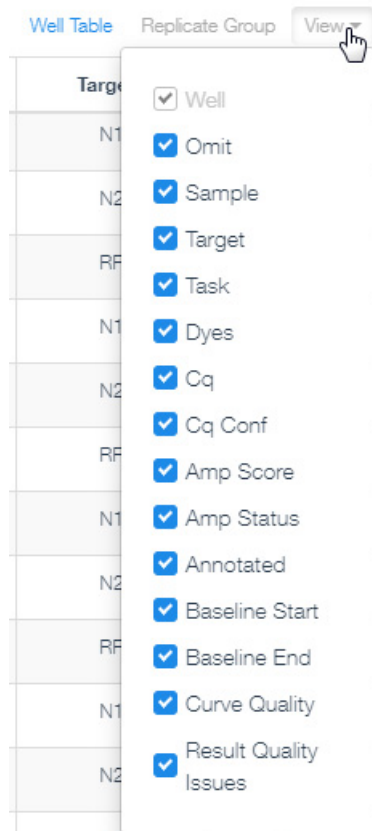
Figure 31 View menu for the Design & Analysis Well Table – default column selection

2 Adjust the settings so that the only columns marked for inclusion are Well, Sample, Target, Task, and Cq. Refer to **Figure 32**.

Figure 32 View menu for the Design & Analysis Well Table – custom column selection

3 Click **View** to collapse the menu.

Step 2. Export the data

4 In the toolbar immediately above the Well Table, click the toolbar button with three dots. ●●●

5 In the menu that opens, click **Export**.

A dialog box opens for saving the CSV file.

6 Select a folder for the file.

7 Into the File name field, type a name for the file.

8 Click **Save**.

The dialog box closes and the program exports the data to the CSV file type and saves it in the designated folder.

9 Open the file and review the Cq data for each of the test samples and controls to interpret the results. See **Chapter 6**, "Analysis and Results."

Perform qRT-PCR on Bio-Rad CFX96 Touch Real-Time PCR Detection System

This section describes the procedure for performing qRT-PCR on a Bio-Rad CFX96 Touch Real-Time PCR Detection System.

Run the qRT-PCR program on the CFX96 Touch Real-Time PCR Detection System

1   Make sure that the CFX96 Touch instrument is powered on.

2   From the instrument-connected PC, open the experiment that you created earlier in the CFX Maestro software application.

3   Navigate to the Start Run tab of the Run Setup screen.

    If the instrument is on but is not displayed under **Block Name**, then click **Detect Instrument(s)** in the top left corner of the screen.

4   In the table, select the instrument to be used for running the experiment.

5   Click **Open Lid**.

    The instrument lid opens.

6   Load your reaction plate into the instrument's thermal block.

7   On the PC, click **Close Lid**.

    The instrument lid closes.

8   Click **Start Run.**

9   Save the run file to the desired folder. Enter a file name and click **Save**.

    The instrument starts running the experiment.

Assign data analysis settings for the CFX96 Touch Real-Time PCR Detection System

Step 1. View the amplification plots for all targets in all wells

1   When the run is complete, open the experiment in the CFX Maestro software application.

    The experiment opens in the Data Analysis window.

2   At the top of the window, click the Quantification tab, if not already selected.

    The window displays the amplification plots, a table of the results in each well, and a diagram of the plate map.

3 Apply the Fluorescence Drift Correction feature.

- At the top of the Data Analysis window, click **Settings > Baseline Settings > Apply Fluorescence Drift Correction**.

  The software automatically corrects any drift in fluorescence signal that may be present in the baseline cycle range.

4 Check for amplification plots with early amplification (i.e., prior to cycle 9). Exclude any test samples with early amplification from the analysis and re-test at a lower concentration.

In test samples with unusually high concentrations of viral RNA, amplification of the viral targets may reach detectable levels at a very early cycle number (<9). As part of the baseline correction calculations, the software may interpret such early amplification signals as background noise and fail to assign a Cq value to the viral targets in those samples.

Monitor for the presence of such test samples by viewing the amplification plots without baseline correction (set **Baseline Setting** to **No Baseline Subtraction**). If any of the test samples do show signs of amplification prior to cycle 9, then remove those wells from the analysis from the Plate Editor window. Re-run the qRT-PCR reaction for the sample by diluting the nucleic acid 1:100 using the appropriate elution buffer.

Step 2. Assess threshold values

5 Check the selection in **Settings > Baseline Setting** to make sure it is set to **Baseline Subtracted Curve Fit**.

6 Right-click on the Amplification graph and select **Show Threshold Values** (if not already marked).

The threshold line for each target is displayed as a horizontal line.

7 Near the bottom right corner of the Amplification graph, mark the Log check box.

Viewing the amplification plots in log values provides a better view of the background signal noise.

8 Set the minimum value for the Y-axis to 0.0.

a To the right of the Amplification graph, click the Chart Settings icon.

The Chart Settings dialog box opens.

b Click the Axes Scale tab.

c Clear the Auto Scale check box.

d In the **Min** column for the **Y-Axis (log 10)**, type **0.0**, as shown in **Figure 33**.

e Click **OK**.

The dialog box closes and the Y-axis scale starts at 0.0, allowing the signal noise in the plots to be displayed. An example is shown in **Table 34**.
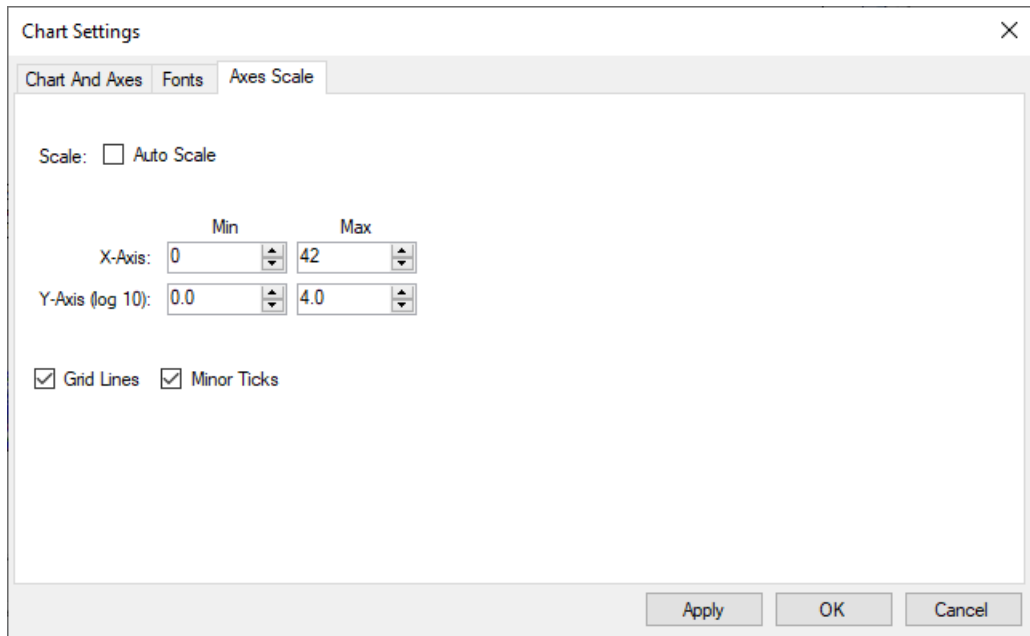
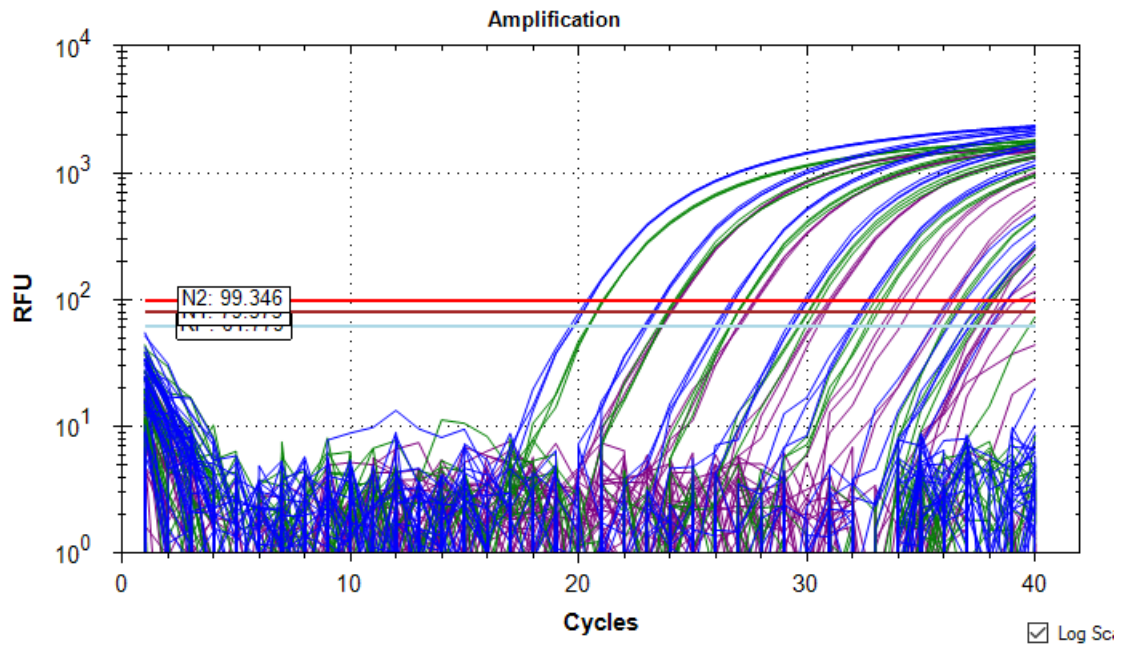**Figure 33**   Maestro Chart Settings dialog box - Axes Scale tab

Figure 34   Maestro Amplification graph in log scale – target thresholds displayed as horizontal
lines

9   Visually assess the amplification plots and default threshold for the N1 target.

   a   Below the Amplification graph, use the check boxes to select only the FAM (N1) target for
display.

b Determine if the threshold is both high enough to be above the background noise and low enough to include plots in the exponential amplification phase (refer to **Figure 35**). Based on this determination, proceed as directed in **Table 15**.

10 Repeat **step 11** for the N2 target and again for the RP target.
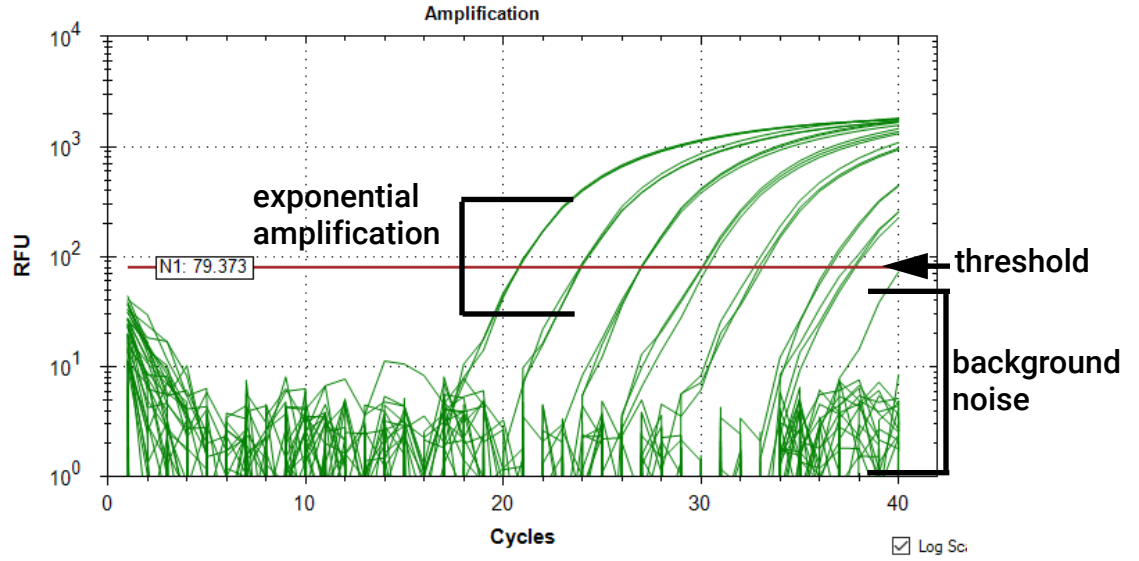
Figure 35 Maestro Amplification graph for N1 target

Table 15 Verifying optimal threshold setting in the Design & Analysis software

Threshold position	Description	Action
Optimal position	Above background noise and includes all plots in exponential amplification phase	• Leave the thresholds at their current values.
Too high	Some plots in exponential amplification phase do not pass threshold	• Look for wells with exceptionally high background noise. Such outlier wells can cause the software to set the threshold too high. • Click **Plate Setup > View/Edit Plate** to open the Plate Editor. • Select the outlier well on the plate map. • In the panel on the right side of the Plate Editor, mark the check box labeled **Exclude Wells in Analysis** (located at the bottom of the panel). • Click **OK**. When asked if you want to apply the changes, click **Yes**.Software will recalculate the threshold once the outlier well is excluded. Alternatively, use the mouse to manually drag the threshold line on the graph to a new position. • On the right side of the Amplification graph, click the Baseline Threshold icon to open the Baseline Threshold dialog box. • Under **Single Threshold**, change the selection from **Auto Calculated** to **User Defined**. (This prevents the software from recalculating the threshold once the outlier well is re-included.) Click **OK** to close the dialog box. • Click **Plate Setup > View/Edit Plate** to re-open the Plate Editor. Select the outlier well, and clear the **Exclude Wells in Analysis** check box. Click **OK** and then **Yes**. • In the plate map under the Amplification graph, select all wells to re-include the outlier well in the analysis.* * If the amplification plot for the outlier well does not show exponential amplification at any cycle number, then make sure that the lowered threshold does not cause the software to assign a Cq value to the well (due to background signal noise crossing the threshold). If it does, then try modifying other analysis settings for that well, e.g., adjusting the Baseline Begin and/or Baseline End cycle numbers.
Too low	Some plots are not above the background noise.	• Increase the threshold to just above the level of background noise.

Step 3. Verify the results in the NTC well

11 In the table displaying the results, locate the No Template Control (NTC) reaction, located in well A12.

12 Make sure that the Cq column for all three targets reads "N/A" or has a Cq value > 37.00.

If the NTC reaction has a Cq value ≤ 37.00 for any of the targets, then sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

Export the data from the CFX Maestro software

1 At the top of the Data Analysis window, click **Export > Custom Export**.

The Custom Export dialog box opens.

2 In the Export Format dialog box, select a file type for the exported data, e.g., **Excel 2007 (*.xlsx)**.

Make sure your PC has the necessary software for opening the selected file type.

3 In the dialog box, mark the check boxes as shown in **Figure 36**. Review the Exported Columns section to make sure it lists **Well**, **Target Name**, **Sample Name**, and **Cq**.
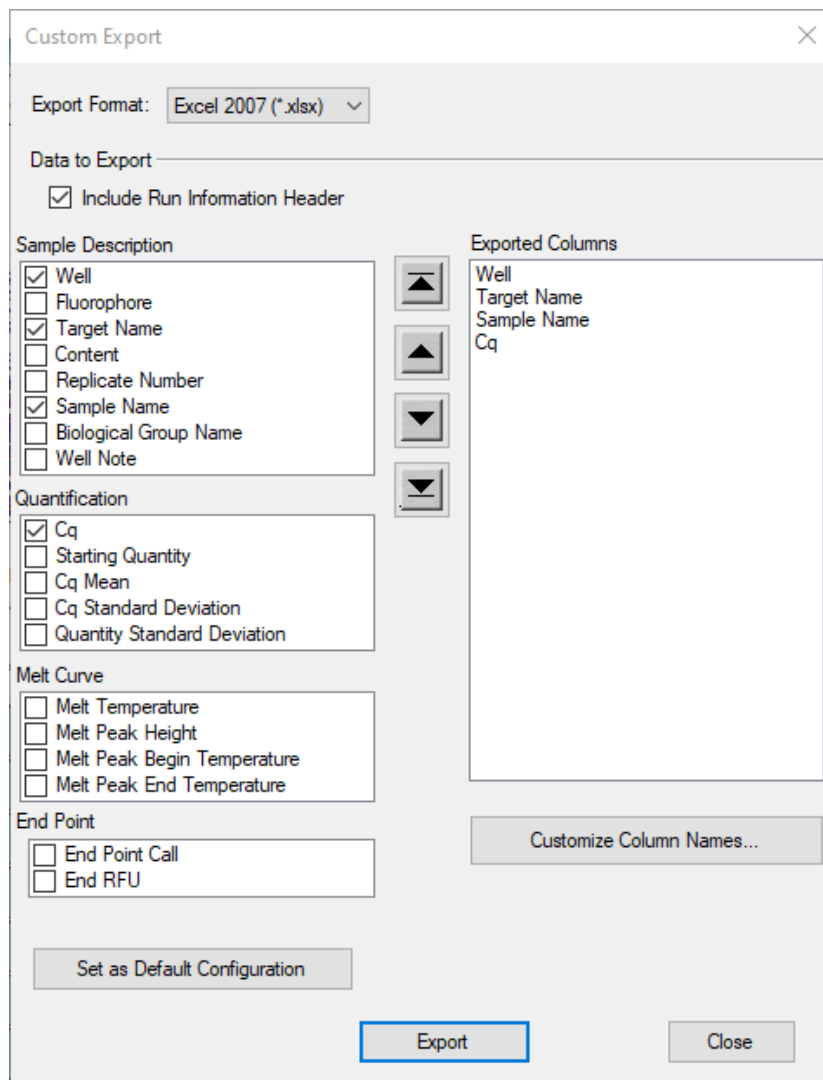
Figure 36 Custom data export settings for the CFX Maestro application

4 Click **Export**.

The Save As dialog box opens.

5 Select a folder for the file.

6 Into the File name field, type a name for the file or use the default.

7 Click **Save**.

The dialog box closes and the program exports the data to the selected file type and saves it in the designated folder.

8 Open the file and review the Cq data for each of the test samples and controls to interpret the results. See **Chapter 6**, "Analysis and Results."

6   Analysis and Results

The chapter contains information on interpreting assay results based on the qRT-PCR data.

Interpretation of Results

Results and interpretation for control samples

If any of the controls do not exhibit the expected performance as described in **Table 16**, then the assay may have been improperly set up or executed or a reagent or equipment malfunction may have occurred. In such a case, invalidate the run and repeat the assay.

No Template Control (NTC)

The NTC consists of using nuclease-free water in the qRT-PCR reaction instead of RNA. The NTC reaction should not exhibit fluorescence growth curves that cross the threshold line within 37.00 cycles for any of the three targets. If the NTC reaction exhibits a growth curve with a Cq ≤ 37.00, then sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

SARS-CoV-2 Synthetic Positive RNA Control Dx

The qRT-PCR reaction for the SARS-CoV-2 Synthetic Positive RNA Control Dx will yield a positive result with the 2019-nCoV_N1 and 2019-nCoV_N2 targets with a Cq value less than or equal to 37.00, while yielding a negative result (reported as no Cq detected or Cq > 37.00) with the human RNase P gene target.

Human Specimen Control

The Human Specimen Control consists of noninfectious human cells and is used as an RNA extraction procedural control to demonstrate successful RNA extraction and extraction reagent integrity. The qRT-PCR reaction for the RNA extracted from the Human Specimen Control should yield a positive result with the RP target with a Cq value less than or equal to 37.00. The N1 and N2 SARS-CoV-2 targets should yield negative results (reported as no Cq detected or Cq > 37.00).

Table 16 Expected Performance of Controls

Control type	Control name	Used to monitor	N1 target result	N2 target result	RP target result
Positive	SARS-CoV-2 Synthetic Positive RNA Control Dx	Substantial reagent failure including primer and probe integrity	Positive (Cq ≤ 37.00)	Positive (Cq ≤ 37.00)	Negative (No Cq detected or Cq > 37.00)
Negative	NTC	Reagent and/or environmental contamination	Negative (No Cq detected or Cq > 37.00)	Negative (No Cq detected or Cq > 37.00)	Negative (No Cq detected or Cq > 37.00)
Extraction	Human Specimen Control	Failure in lysis and extraction procedure, potential contamination during extraction	Negative (No Cq detected or Cq > 37.00)	Negative (No Cq detected or Cq > 37.00)	Positive (Cq ≤ 37.00)

Results and interpretation for clinical samples

Table 17 lists the expected results for the assay. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please contact CDC for consultation and possible specimen referral.

Human RNase P gene (RP) target

All clinical samples should exhibit fluorescence growth curves for the RP target that cross the threshold line within 37.00 cycles (Cq ≤ 37.00), thus indicating the presence of the human RNase P gene. Failure to detect the RP target in any clinical samples may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation or carryover of inhibitory substances.
- Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
- Improper assay set up and execution.
- Reagent or equipment malfunction.

If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:

- If the N1 and N2 targets are positive (Cq ≤ 37.00) even in the absence of a positive RP, then the result should be considered valid. It is possible that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample or due to viral targets reducing RP amplification efficiency, especially in specimens with high viral load. A negative RP signal does not preclude the presence of SARS-CoV-2 virus RNA in a clinical specimen.
- If the RP target is negative (no Cq detected or Cq > 37.00) while one or both of the N1 and N2 targets are negative (no Cq detected or Cq > 37.00), then the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all targets remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

2019-nCoV_N1 and 2019-nCoV_N2 targets

- When all controls exhibit the expected performance, a specimen is considered **positive** for SARS-CoV-2 if the following condition is met.
  - Growth curves for both the N1 and N2 targets cross the threshold line within 37.00 cycles (Cq ≤ 37.00). The RP target may or may not be positive (see description above) but the SARS-CoV-2 result is still valid.
- When all controls exhibit the expected performance, a specimen is considered **negative** for SARS-CoV-2 if BOTH of the following conditions are met.
  - Growth curves for the N1 and N2 targets DO NOT cross the threshold line within 37.00 cycles (no Cq detected or Cq > 37.00).
  - Growth curve for the RP target DOES cross the threshold line within 37.00 cycles (Cq ≤ 37.00).
- When all controls exhibit the expected performance, a specimen is considered **inconclusive** for SARS-CoV-2 if EITHER of the following conditions are met.
  - Growth curve for one of the 2019-nCoV targets crosses the threshold line within 37.00 cycles (Cq ≤ 37.00) while the growth curve for the other 2019-nCoV target does not cross the threshold line within 37.00 cycles (no Cq detected or Cq > 37.00). The growth curve for the RP target crosses the threshold line within 37.00 cycles (Cq ≤ 37.00).

For inconclusive specimens, the extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, report the inconclusive result.

- If the Human Specimen Control is positive for N1 or N2 (Cq ≤ 37.00), then contamination may have occurred during extraction or sample processing. Invalidate all results for specimens extracted alongside the Human Specimen Control. Re-extract specimens and the Human Specimen Control and re-test.

Table 17 Expected results for the SARS-CoV-2 qRT-PCR assay

2019-nCoV_N1	2019-nCoV_N2	Human RNase P gene	Result interpretation*	Report	Actions
Positive (Cq ≤ 37.00)	Positive (Cq ≤ 37.00)	Positive OR Negative	SARS-CoV-2 detected	Positive SARS-CoV-2	Report results to CDC and sender
Negative (No Cq detected or Cq > 37.00)	Negative (No Cq detected or Cq > 37.00)	Positive (Cq ≤ 37.00)	SARS-CoV-2 not detected	Not detected	Report results to sender. Consider testing for other respiratory viruses.[†]
Positive (Cq ≤ 37.00)	Negative (No Cq detected or Cq > 37.00)	Positive (Cq ≤ 37.00)	Inconclusive result	Inconclusive	Repeat testing of extracted sample and/or repeat extraction and qRT-PCR.
Negative (No Cq detected or Cq > 37.00)	Positive (Cq ≤ 37.00)	Positive (Cq ≤ 37.00)	Inconclusive result	Inconclusive	Repeat testing of extracted sample and/or repeat extraction and qRT-PCR.
If one or both of the 2019-nCov targets is negative (no Cq detected or Cq > 37.00)	Negative (No Cq detected or Cq > 37.00)		Invalid result	Invalid	Repeat extraction and qRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

* Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

[†] Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.

7   Quality Control

This chapter contains information on quality control measures for the assay.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory's standard quality control procedures.
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- The Human Specimen Control **must** proceed through nucleic acid extraction with each batch of specimens to be tested.
- **Always** include a no template control (NTC) and the SARS-CoV-2 Synthetic Positive RNA Control in each amplification and detection run.
- The probe/primer set for the human RNAse P gene (included in the 10× SARS-CoV-2 Primer/Probe Mix) controls for specimen quality and extraction.

8   Limitations of the Assay

This chapter describes the limitations of the assay performed with the Agilent SARS-CoV-2 qRT-PCR Dx Kit

Limitations

1 Use of this assay is limited to personnel who are trained in the procedure. Failure to follow these instructions may result in erroneous results.

2 Reliable results are dependent on adequate specimen collection, transport, storage, and processing.

3 Avoid contamination by adhering to good laboratory practices and to the procedures specified in this instructions for use.

4 Negative results do not preclude SARS-CoV-2 infections and should not be used as the sole basis for treatment or other management decisions.

5 A positive result indicates the detection of nucleic acid from the relevant virus. Nucleic acid may persist even after the virus is no longer viable.

6 The performance of the Agilent SARS-CoV-2 qRT-PCR Dx Kit assay was established using nasopharyngeal swab specimen type collected in UTM or VCM transport media. Oropharyngeal swabs, nasal swabs, and mid-turbinate nasal swabs are considered acceptable specimen types for use with the Agilent SARS-CoV-2 qRT-PCR Dx Kit assay but performance with these specimen types has not been established. Testing of oropharyngeal swabs, nasal swabs, and mid-turbinate nasal swabs (self-collected under supervision of or collected by a healthcare provider) is limited to patients with symptoms of COVID-19.

7 The Agilent SARS-CoV-2 qRT-PCR Dx Kit does not include a sample for use as the Human Specimen Control. The sample used as this control must be validated by the laboratory.

9 Performance Characteristics

This chapter contains the performance characteristics of the Agilent SARS-CoV-2 qRT-PCR Dx Kit.

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Performance Characteristics

The following data demonstrate the performance characteristics of the Agilent SARS-CoV-2 qRT-PCR Dx Kit. All sample extractions used the specimen input volumes and elution volumes recommended in the **"Extract Nucleic Acids"** section on **page 19**.

Analytical Sensitivity (Limit of Detection)

Agilent conducted a Limit of Detection (LoD) study to establish the lowest SARS-CoV-2 viral concentration (expressed as the number of viral genome copies) that can be detected by the Agilent SARS-CoV-2 qRT-PCR Dx Kit at least 95% of the time with each of the supported nucleic acid extraction procedures on each of the three supported real-time PCR systems.

The LoD testing sample panels were contrived by diluting a quantified positive clinical specimen with pooled negative clinical specimens (negative matrix) to achieve a series of desired SARS-CoV-2 target concentrations. All clinical specimens were nasopharyngeal swabs previously tested for SARS-CoV-2 by the vendor using a SARS-CoV-2 test with Emergency Use Authorization and further confirmed by Agilent.

The two preliminary whole assay LoD experiments used a range of viral concentrations with three replicates per concentration. In the first preliminary experiment, all of the viral concentrations in the dilution series (ranging from 0.5 to 3 copies/μl) were determined to be below the LoD. The concentration range was adjusted in the second preliminary experiment to test concentrations from 4 to 15 copies/μl. The results of the second preliminary experiment indicated that the final LoD would likely fall withing this range and vary depending on extraction method and real-time PCR system.

The final whole assay LoD confirmation experiment was conducted on three target input levels: 4, 8, and 16 copies/μl. At each of the three target input levels, 20 individual extraction replicates were tested across all of the possible extraction method/real-time PCR system combinations. **Table 18** shows the positive detection rates from that experiment. A detection rate of 20/20 indicates that all 20 extraction replicates were detectable with that particular combination of extraction method and real-time PCR system.

Table 19 summarizes the final LoD values for each extraction method/real-time PCR system combination. These values represent the number of viral copies per μl of nucleic acid extract that can be detected by the Agilent SARS-CoV-2 qRT-PCR Dx Kit at least 95% of the time.

Table 18 Final Limit of Detection Confirmation -- Positive detection rates* with each nucleic acid extraction method on each real-time PCR system (Agilent AriaMx, ABI 7500, and Bio-Rad CFX96)

SARS-CoV-2 viral target input level prior to extraction (copies/µl)	QIAsymphony DSP Virus/Pathogen Midi Kit with QIAsymphony (automated extraction)			MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit with KingFisher Flex (automated extraction)		
	AriaMx	**7500**	**CFX96**	**AriaMx**	**7500**	**CFX96**
16	20/20	20/20	20/20	20/20	20/20	20/20
8	7/7 (13)	14/14 (6)	9/11 (9)	17/17 (3)	19/19 (1)	19/20
4	2/4 (16)	8/11 (9)	10/13 (7)	19/19 (1)	8/9 (11)	19/19 (1)
0 (blank)	0/2	0/2	0/2	0/2	0/2	0/2

* Detection rates are expressed as the number of positive replicates out of the total number of positive and negative replicates. Numbers in parentheses, when present, indicate the number of inconclusive replicates. In these cases, the total number of positive and negative replicates is less than 20.

Table 19 Final Limit of Detection Summary -- LoD for each nucleic acid extraction method on each real-time PCR system (Agilent AriaMx, ABI 7500, and Bio-Rad CFX96)

Nucleic acid extraction method	Whole Assay Limit of Detection (copies/µl)		
	AriaMx	**7500**	**CFX96**
QIAsymphony DSP Virus/Pathogen Midi Kit with QIAsymphony (automated extraction)	16	16	16
MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit with KingFisher Flex (automated extraction)	16	8	4

Analytical Inclusivity

In the Agilent SARS-CoV-2 qRT-PCR Dx Kit, the sequences of the oligonucleotide primers and probes for the N1 and N2 viral targets and the human RNase P gene are identical in sequence to those used in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel for Emergency Use Only. Inclusivity of this panel has been previously established.

Cross-Reactivity

In the Agilent SARS-CoV-2 qRT-PCR Dx Kit, the sequences of the oligonucleotide primers and probes for the N1 and N2 viral targets and the human RNase P gene are identical in sequence to those used in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel for Emergency Use Only. Cross-reactivity of this panel has been previously established.

Clinical Evaluation

A clinical evaluation study was conducted with the Agilent SARS-CoV-2 qRT-PCR Dx Kit using human nasopharyngeal swab (NP) specimens. For each test condition, a total of 80 specimens were tested: 40 negative NP specimens and 40 positive NP specimens. The positive or negative status for each specimen was confirmed by an Emergency Use Authorization (EUA) qRT-PCR assay for SARS-CoV-2. The objective of the clinical evaluation was to asses the clinical agreement (positive/negative) of test results obtained with the Agilent SARS-CoV-2 qRT-PCR Dx Kit to those obtained with the comparator EUA qRT-PCR assay for SARS-CoV-2.

Nucleic acid extraction was performed on each specimen using both of the supported automated extraction platforms (QIAGEN QIAsymphony DSP Virus/Pathogen Midi Kit with the QIAsymphony SP and Thermo Fisher Scientific MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit with the KingFisher Flex Purification System) using the manufacturer's protocol and the recommendations provided in these Instructions for Use. Each extracted sample was then tested on all three of the supported real-time PCR systems (Agilent AriaMx, ABI 7500, and Bio-Rad CFX96) as described in these Instructions for Use. Thus, each specimen was tested under six (6) different conditions in this study. As acceptance criteria, a minimum of 95% positive and negative agreement was required between the Agilent SARS-CoV-2 qRT-PCR Dx Kit and the comparator assay. The agreement results are summarized in **Table 20**.

Table 20 Positive percent agreement (PPA), negative percent agreement (NPA), and overall agreement (OA) between Agilent SARS-CoV-2 qRT-PCR Dx Kit and comparator EUA qRT-PCR assay for SARS-CoV-2

Extraction platform:	QIAsymphony	QIAsymphony	QIAsymphony	KingFisher	KingFisher	KingFisher
Real-time PCR system:	**AriaMx**	**7500**	**CFX96**	**AriaMx**	**7500**	**CFX96**
PPA	97.4%	100.0%	100.0%	97.5%	97.5%	97.5%
NPA	100.0%	97.5%	100.0%	97.5%	97.5%	97.5%
OA	98.7%	98.8%	100.0%	97.5%	97.5%	97.5%

10 Reference

Product Labels   **78**

This chapter contains copies of the product labels for the Agilent SARS-CoV-2 qRT-PCR Dx Kit.

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Product Labels

Figure 37   Box label for K1180-64100

Figure 38   Bottle label for 5191-6866

Figure 39   Tube label for 5191-6870

Reference Dye Dx CE [IVD]

[REF] 5191-6871 -25°C ⟍ -15°C
[LOT] ABC123
100 µl 1 mM ⧗ 2020-12-31
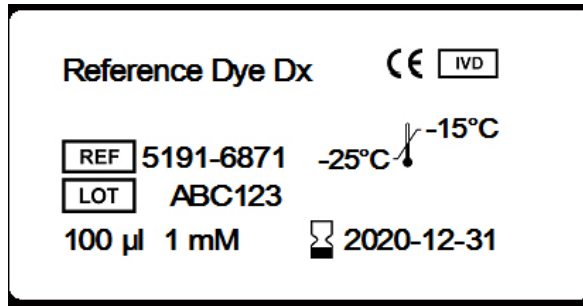
Figure 40 Tube label for 5191-6871

100 mM DTT Dx CE [IVD]

[REF] 5191-6872 -25°C ⟍ -15°C
[LOT] ABC123
100 µl ⧗ 2020-12-31
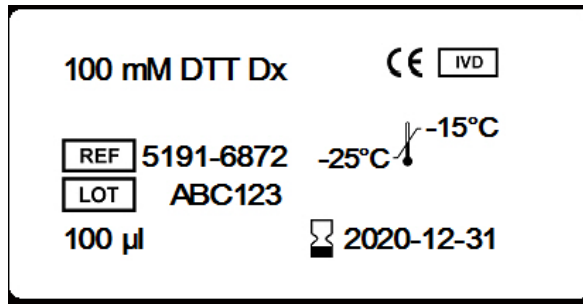
Figure 41 Tube label for 5191-6872

10X SARS-CoV-2 CE [IVD]
Primer/Probe Mix Dx ⟍ -15°C
[REF] 5191-6867 -25°C
[LOT] ABC123
800 µl ⧗ 2020-12-31
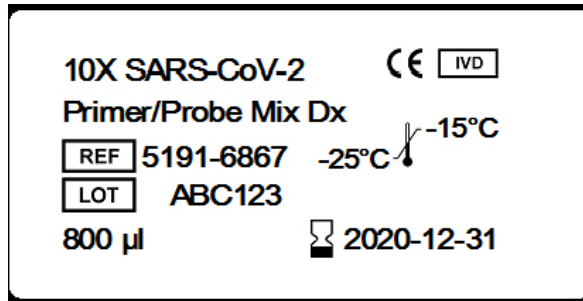
Figure 42 Tube label for 5191-6867

Figure 43 Box label for K1180-64200

Figure 44 Tube label for 5191-6868

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