AMPIPROBE® SARS-CoV-2 Test System (RUO)

INSTRUCTIONS FOR USE

Multiplex real-time RT-PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2

ENZ-GEN225-0100 [AMPIXTRACT™ SARS-CoV-2 Extraction (RUO), 1 x 96 samples]
ENZ-GEN230-0100 [AMPIPROBE® SARS-CoV-2 Assay (RUO), 1 x 96 samples]
ENZ-GEN231-0004 [AMPIPROBE® SARS-CoV-2 Controls (RUO), 1 x 4 samples]

For the latest product information, including support documentation, visit us online:
www.enzolifesciences.com

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INTENDED USE

The AMPIPROBE® SARS-CoV-2 Test System (RUO) is a multiplex assay system that is based on real-time reverse transcription polymerase chain reaction (rRT-PCR), intended for the qualitative detection of acute upper respiratory SARS-CoV-2 specific RNA.

For research use only (RUO)! Not for use in diagnostic procedures.

PRODUCT DESCRIPTION

The AMPIPROBE® SARS-CoV-2 Test System includes the following components:

- AMPIXTRACT™ SARS-CoV-2 Extraction kit (RUO)
- AMPIPROBE® SARS-CoV-2 Assay kit (RUO)
- AMPIPROBE® SARS-CoV-2 Controls kit (RUO)

The AMPIXTRACT™ SARS-CoV-2 Extraction kit (RUO) utilizes magnetic-particle technology for automated isolation and purification of nucleic acids from various biological specimens. The AMPIXTRACT™ SARS-CoV-2 Extraction kit (RUO) can be used with manual or automated method. For the automated extraction method, any open liquid handling platform can be used. This kit provides for 100 sample extractions. The following protocols were validated with this kit:

- Automated Method Using the GENFLEX™ Platform (open platform), refer to section “RNA Extraction and Preparation of RT-PCR Reactions (Automated Method Using the GENFLEX™ Platform)”
- Manual Method, refer to section “RNA Extraction and Preparation of RT-PCR Reactions (Manual Method)”

The AMPIPROBE® SARS-CoV-2 Assay (RUO) is a multiplexed real-time RT-PCR assay that contains two primer and probe sets to detect two regions in the SARS-CoV-2 nucleocapsid (N) gene and one primer and probe set to detect human RNase P (RP) in samples. RNA isolated from upper respiratory specimens is reverse transcribed to cDNA and subsequently amplified using any qPCR instrument, such as the Applied Biosystems QuantStudio® 5 (QS5). During the amplification 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 bound probe, causing the reporter dye (FAM, Hex, and Quasar 670) to separate from the quencher dye (BHQ1 or BHQ2), generating a fluorescent signal. Fluorescence intensity is monitored at each PCR cycle by the thermocycler. Each AMPIPROBE® SARS-CoV-2 Assay kit provides reagents for 100 assays.

The AMPIPROBE® SARS-CoV-2 Controls kit (RUO) contains a positive RNA control that is specific to the SARS-CoV-2 genomic regions targeted by the assay as well as human RNase P, a negative RNA control that contains target to human RNase P, and a no-template control (NTC). Each kit provides control reagents for 4 runs. This kit can be used with any qPCR instrument.

KIT CONTENTS AND STORAGE

Store all unopened reagents and controls as recommended in the following tables. When stored properly, the reagents are stable until the expiration date indicated on the labels.

AMPIXTRACT™ SARS-CoV-2 Extraction Kit (RUO) (ENZ-GEN225-0100)[a]			
Components		**Quantity**	**Storage**
AMPIXTRACT™ Wash Reagent		1 x 50 mL	RT (20-25ºC)
AMPIXTRACT™ Lysis Reagent		1 x 80 mL	RT (20-25ºC)
AMPIXTRACT™ Elution Buffer		1 x 12 mL	2-8ºC
AMPIXTRACT™ Magnetic Beads		1 x 5 mL	2-8ºC
AMPIXTRACT™ PK Dilution Buffer		1 x 9 mL	2-8ºC
SPC Pack	AMPIXTRACT™ Stabilizer	2 x 900 µL	-20ºC
	AMPIXTRACT™ Proteinase	1 x 350 µL	-20ºC
	AMPIXTRACT™ Carrier RNA	1 x 1.2 mL	-20ºC

[a] This kit can be ordered as a stand-alone product.

AMPIPROBE® SARS-CoV-2 Assay (RUO) (ENZ-GEN230-0100)[a]		
Components	**Quantity**	**Storage**
AMPIPROBE® RT-PCR Buffer	1.3 mL	-20ºC
AMPIPROBE® SARS-CoV-2 Primer/Probe Mix	260 µL	-20ºC
AMPIPROBE® RT-PCR Enzyme Mix	130 µL	-20ºC
Nuclease-free Water	1 mL	-20ºC

[a] This kit can be ordered as a stand-alone product.

AMPIPROBE® SARS-CoV-2 Controls Kit (RUO) (ENZ-GEN231-0004)[a]		
Components	**Quantity**	**Storage**
AMPIPROBE® SARS-CoV-2 Positive Control	100 µL	-80ºC
AMPIPROBE® SARS-CoV-2 Negative Control	100 µL	-80ºC
No Template Control (NTC)	100 µL	-80ºC

[a] This kit can be ordered as a stand-alone product.

REQUIRED MATERIALS NOT PROVIDED (to be supplied by the user)

1. Components required but not included with the AMPIPROBE® SARS-CoV-2 Test System for AUTOMATED extraction and PCR setup using the GENFLEX™ platform

Instruments and Equipment

a. 200 mL Trough (Hamilton 56695-01)

b. 20 mL Trough (Hamilton 96424-02)

c. Plastic waste chute (Hamilton 185319)

d. Waste container for hazardous waste (Hamilton 281520)

e. Biohazard plastic liner for waste container (Hamilton 53686-01)

f. Vortex mixer

g. Microcentrifuge

h. GENFLEX™ Platform (ENZ-GEN-FLX), includes a liquid handler used for automated RNA extraction and PCR reaction setup, and QuantStudio® 5 Real-Time PCR System (Applied Biosystems A28139), 96-well, with QuantStudio® Design & Analysis Desktop Software version 1.5.1

Consumables

a. Deep-well plates (DWP) (Thomas Scientific 1162C08)

b. Elution microplate (Thomas Scientific 1144B80)

c. 96-well PCR Plate (DN Biotech 5371009 or equivalent)

d. Optical Adhesive Film, for use in PCR instrument

e. Adhesive film, for storing RNA plate

f. Small reagent tubes (Sarstedt 72.703 or equivalent)

g. 1 mL tips (Hamilton 235940)

h. 300 µL tips (Hamilton 235938)

Reagents

a. 100% Ethanol, Molecular Biology Grade (Sigma E7023 or equivalent)

2. Components Required but not included with the AMPIPROBE® SARS-CoV-2 Test System for MANUAL extraction and PCR setup using the GENFLEX™ platform

Instruments and Equipment

a. Plate magnet (e.g. Alpaqua Enhanced Universal Magnet Plate, Alpaqua # A000400), for RNA extraction

b. Heater shaker (e.g. Bull Dog # 1808-0506), for RNA extraction

c. Real-Time PCR System capable of reading FAM, HEX and Cy5 (e.g. QuantStudio® 5 Real-Time PCR System, Applied Biosystems A28139)

Consumables

a. Deep-well plates (DWP)

b. 96-well PCR Plate

c. Optical Adhesive Film, for use in PCR instrument

Reagents

a. 100% Ethanol, Molecular Biology Grade

WARNINGS AND PRECAUTIONS

1. This product is for research use only.
2. The SARS-CoV-2 assay should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of samples and controls to prevent false positive results. Samples and reagents must be handled under a laminar airflow hood or biological safety cabinet.
3. Kit components should be stored at the right temperatures as indicated on the labels. Care should be taken to limit the number of freeze-thaw cycles where applicable.
4. Always check the expiration date prior to use of reagents. DO NOT use expired reagents.
5. When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles.
6. Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
7. Handle all samples and controls as if they are capable of transmitting infectious agents. Positive results are indicative of the presence of SARS-CoV-2 RNA.

8. Some of the AMPIXTRACT™ SARS-CoV-2 Extraction kit (RUO) components contain hazardous substances. The Lysis Reagent, which contains sodium perchlorate, and ethanol, and Proteinase can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Consult the appropriate material safety data sheets (MSDS) for more information.
9. If liquid containing the buffers is spilt, clean with suitable laboratory detergent and water. Specifically, the AMPIXTRACT™ Lysis Reagent contains sodium perchlorate, a corrosive. Spills of this reagent should be avoided. If this reagent is spilt, clean up the instrument or surface with 70% ethanol.
10. If liquid containing potentially infectious agents is spilt, clean the area first with laboratory detergent and water**,** and then with 10% (v/v) sodium hypochlorite followed by 70% ethanol.
11. Practice aseptic technique when handling reagents to avoid introduction of contaminants that might interfere with assay interpretation.
12. The use of screw-cap tubes and barrier pipette tips is strongly encouraged to prevent samples and reagents from becoming aerosolized which might lead to contamination.
13. Avoid exposure of reagents to UV light (used for decontamination) or continuous exposure to light, especially the fluorogenic reagents. It may cause accelerated aging of the reagents and buffers.
14. Observe good laboratory practices. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. Any biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

QUALITY CONTROL

CONTROLS

The following controls provided in the AMPIPROBE® SARS-CoV-2 Controls (RUO) kit, ENZ-GEN231-0004 (RUO), must be included for each run to accurately interpret results:

- One Positive Control (PC) for PCR that contains SARS-CoV-2 targets N1 and N2, and human RNase P target.
- One Negative Control (NC) for PCR that contains human RNase P target.
- One No Template Control (NTC) that do not contain any targets.

Note: *Internal Control (IC), which is human RNase P, is present in human specimens. The SARS-CoV-2 assay utilizes a sequence from the human RNase P gene as the IC target. The presence of human RNase P should be detected in every specimen, and in Extraction Controls (EC), and Positive and Negative Controls (PC and NC).The internal control verifies that nucleic acid is present in the sample and is used for every sample processed. This is also used as the extraction control to ensure that samples resulting as negative contain nucleic acid for testing.*

Positive Controls (PC). The PC is the *in vitro* transcribed and purified viral SARS-CoV-2 RNA targets N1 and N2 and is needed to verify that the assay run is performing as intended. It is used in every RT-PCR assay plate starting at master mix addition, and 12 µL of PC would provide 108 copies in the PCR reaction and is equivalent to 1544 copies/ml of starting material, which is less than 5x LoD. The positive control also includes an RNase P target and provides 2160 copies in PCR and will result as "Positive" for that marker.

Negative Control (NC). The NC is needed to verify that the assay run is performing as intended and is used on every assay plate starting at the master mix addition with no SARS-CoV-2 RNA target, but with *in vitro* transcribed RNase P target at 2160 copies in PCR and will result as "Negative" for SARS-CoV-2.

No Template Control (NTC). The NTC is needed to eliminate the possibility of sample contamination on the assay run and is used in every RT-PCR assay plate.

In addition to the above, an **Extraction Control (EC)** can also be included. The Extraction Control (EC) to use is a previously confirmed negative sample that is taken through every extraction procedure. The presence of human RNase P should be detected in the extraction control. It serves as a negative control to monitor for any cross-contamination that occurs during the extraction process. Also, it validates successful RNA extraction and validation of extraction reagents.

CORRECTIVE ACTION FOR OUT-OF-CONTROL RESULTS

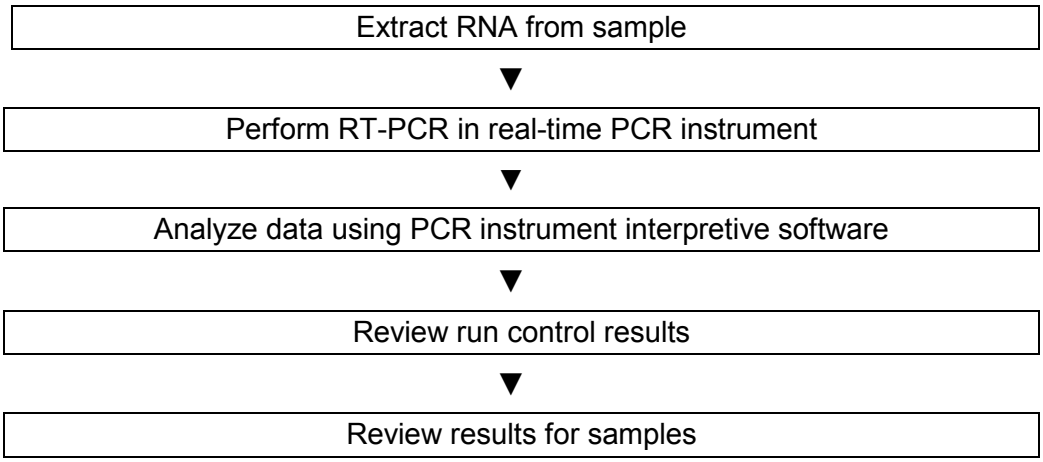
1. If any control (PC, NC, or NTC) result is invalid, the PCR run is invalid.
2. Corrective action, including reviewing sample processing workflow, decontamination of bench and/or workstations, repeat testing of QC and samples, and consultation with kit and instrument manufacturers, should be taken. In general, if the controls are out of their expected range, all of the specimens and controls from that run must be processed beginning from the sample preparation step.

SPECIMEN REQUIREMENT / TYPES / COLLECTION

Handle all specimens and controls as if they are capable of transmitting infectious agents.

Upper respiratory specimens are collected in transport medium. The BD Universal Viral Transport Medium with 3 ml collection media (BD # 220527) **or equivalent** collection device are acceptable for the SARS-CoV-2 assay. Refer to the manufacturer's manual for detailed procedures for collecting samples.

WORKFLOW



The workflow starts with the extraction of the RNA from upper respiratory specimens. RNA is isolated and purified from the specimen using the AMPIXTRACT™ SARS-CoV-2 Extraction Kit (RUO) (Enzo ENZ-GEN225-0100). RNA isolation can be performed manually with the AMPIXTRACT™ SARS-CoV-2 Extraction kit or via an automated process such as Enzo's GENFLEX™ platform. For more information about using the GENFLEX™ platform, see section **RNA EXTRACTION and PREPARATIONS of RT-PCR REACTIONS (Automated Method Using the Enzo GENFLEX™ Platform)**. The AMPIXTRACT™ SARS-CoV-2 Extraction kit (RUO) utilizes magnetic-particle technology for isolation and purification of nucleic acids from upper respiratory specimens. Magnetic-particle technology enables purification of high-quality nucleic acids that are free of proteins, nucleases, and other impurities.

The purified RNA is reversed transcribed into cDNA and amplified using the AMPIPROBE® SARS-CoV-2 Assay kit (RUO) (Enzo ENZ-GEN230-0100) in the real-time PCR instrument, e.g. Applied Biosystems QuantStudio® 5 Real-Time PCR Instrument (AB # A28569).

The data are analyzed, then interpreted by the RT-PCR instrument software.

REAGENTS AND SAMPLE PREPARATION

EXTRACTION REAGENTS PREPARATION

1. **AMPIXTRACT™ Wash Reagent Working Solution**

   Add 200 mL of 100% ethanol to each bottle containing the Wash Reagent solution. Store at room temperature until use.

2. **AMPIXTRACT™ Lysis Reagent Working Solution**

   Add 40 mL of 100% ethanol to each bottle containing the Lysis Reagent solution. Mix thoroughly before use. Store at room temperature until use.

3. **AMPIXTRACT™ Stabilizer**

   Place the vial(s) of Stabilizer on the GENFLEX™ platform after the reagent is completely thawed.

SAMPLE PREPARATION

1. Bring the samples to room temperature. Prepare the sample collection tubes in the BSL2 cabinet. Carefully uncap the sample tubes, and while holding the cap above the vial, carefully remove the swabs from the samples using a long forceps and discard the swabs into a biohazard waste container that contains 10% bleach solution.
2. Proceed to RNA extraction and process the samples according to the RNA extraction method of choice.

RNA EXTRACTION AND PREPARATION OF RT-PCR REACTIONS
(Automated Method Using the Enzo GENFLEX™ Platform)

The following procedures are for the automated RNA Extraction and RT-PCR reaction using Enzo's GENFLEX™ platform (ENZ-GEN-FLX). The GENFLEX™ platform includes a liquid handling workstation (Hamilton Microlab STAR™) and a RT-PCR instrument (Applied Biosystems QuantStudio® qPCR). For other automated methods, refer to the manufacturer's user manual.

PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Thoroughly clean all work surfaces with fresh 10% bleach followed by fresh 70% alcohol before and after each use.
3. The GENFLEX™ platform should be checked prior to daily runs. Documentation of these procedures is logged in the individual instruments' maintenance folder. We recommend following the maintenance instructions given in the user manual

to reduce the risk of contamination.

4. It is recommended to power on the GENFLEX™ platform, along with the external Inheco heating unit, before sample and reagent preparation is started so that the initialization process is complete before the instrument is needed.
5. Before starting a run, ensure that you have sufficient reagents for the batches you are planning to run.
6. Using extraction kits in combination with amplification systems that use extraction control require inclusion of this control into the purification procedure to monitor the efficiency of sample preparation and downstream assay.
7. Since small amounts of liquid are lost during transfer and contact with the magnetic particles, the initial volume of elution solution must be larger than the selected volume to ensure that the final eluate is of the correct volume.
8. Ribonucleases (RNases) are very stable and active enzymes generally do not require cofactors to function. Since RNases are difficult to inactivate and only minute amounts are sufficient to destroy RNA, do not use any plastic ware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure.
9. PCR should be set up immediately after extraction of RNA. For short term or long-term storage, we recommend storage at <-70ºC avoiding repeated freeze-thaw cycles.

EXTRACTION PROCEDURE

Sample Preparation for Extraction

1. Place the Extraction Control (EC), a previously confirmed negative sample, in the sample carrier position **1**.
2. After the swabs have been removed from the sample vials, place the specimen tubes with at least 1mL of the specimen, starting at position **2** of the sample carrier. Load all the sample tubes onto additional sample carriers, if necessary.
3. Once the samples including the control are placed in the carrier with lids removed, bring them carefully from the BSL2 cabinet to the GENFLEX™ platform for loading procedure.

Preparing the Reagents for the Run

1. Unwrap the 200 mL and 20 mL troughs before use. Refer to **Table 1** below.

TABLE 1. Working Solutions and Trough Size	
Working Solutions to be Loaded	**Trough size**
AMPIXTRACT™ Wash Reagent	200 mL
AMPIXTRACT™ Lysis Reagent	200 mL
AMPIXTRACT™ PK Dilution Buffer	20 mL
AMPIXTRACT™ Magnetic Beads	20 mL
AMPIXTRACT™ Elution Buffer	20 mL

2. Transfer the reagents from the bottles provided in the kit to the troughs following the prompts as they appear on the instrument's screen.

Extraction Procedure on the GENFLEX™ Platform

Note: *The sample input volume for extraction is 350 µL and the nucleic acid eluate volume is 60 µL.*

1. Power on the instrument, if not already done so. The power switch (green switch) is located at the bottom-left corner of the instrument.
2. Turn on the Inheco heating unit after the GENFLEX™ platform is turned on. The power switch for the heating unit is located at the back of the unit.
3. Click on the "Method Manager" icon on the desktop.
4. Select COVID-19 icon in red from the list that appears on the instrument's screen.
5. Click on the green "Run" icon next to the COVID-19 method to initiate the run.
6. Once the instrument has finished its startup protocol and initialization, a user prompt box will be displayed within an additional window named "Run Control". Select "Extraction" on the User Input window. Click "continue" and in the next window, choose "96" well format for the PCR setup.
7. In the next screen, select "Primary Tube" as the sample type, input the number of samples loaded onto the instrument, including the extraction control. Stabilizer and Carrier RNA addition are essential for COVID-19 extraction and addition of these reagents needs to be selected.
8. Follow instructions that appear next on the screen. The screen will display a checklist of reagents that needs to be loaded onto the deck of the GENFLEX™ platform as shown in **Figure 1**. This checklist also serves as instructions on the transfer of each reagents to troughs and placement of the troughs in specific locations along given tracks. In addition, it provides information on the placement of the Stabilizer, Carrier RNA and Proteinase tubes. The tubes containing

Stabilizer, Carrier RNA and Proteinase vials from the kit can be directly placed on the deck at correct positions as indicated on the reagent checklist for placements.

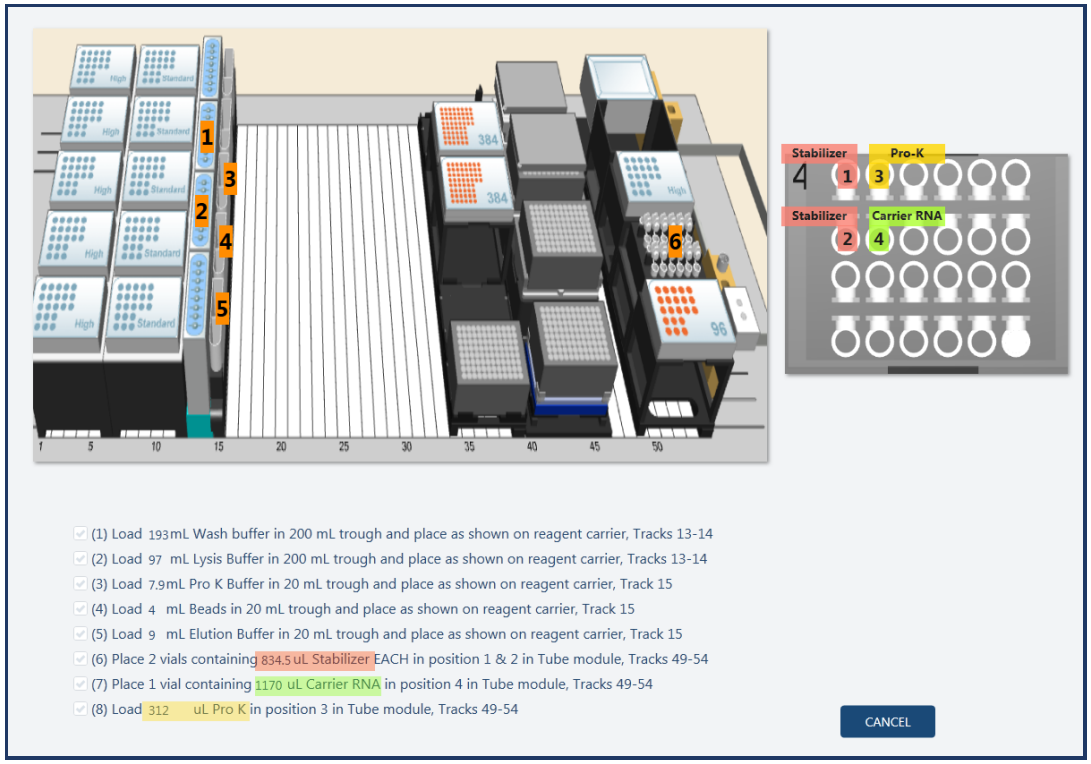


Figure 1. Deck layout diagram for reagent input as shown in the GENFLEX™ Venus Software.

9. **Figure 1** details the individual loading positions highlighted in red. Load the individual troughs with the appropriate volumes of the extraction kit reagents as indicated in **Table 2** below for 96 samples.

TABLE 2. Volume of Extraction Kit Reagents to be Loaded into individual Troughs

Loading Positions	Working Solutions to be Loaded	Volume Loaded	Track #s
1	Pre-diluted AMPIXTRACT™ Wash Reagent	193 mL	13-14
2	Pre-diluted AMPIXTRACT™ Lysis Reagent	97 mL	13-14
3	AMPIXTRACT™ PK Dilution Buffer	7.9 mL	15
4	AMPIXTRACT™ Magnetic Beads	4 mL	15
5	AMPIXTRACT™ Elution Buffer	9 mL	15
6	AMPIXTRACT™ Stabilizer	0.850 mL x 2 vials	49-54
7	AMPIXTRACT™ Proteinase	0.312 mL	49-54
8	AMPIXTRACT™ Carrier RNA	1.2 mL	49-54

10. Once the reagents are loaded as indicated, and the list is checked, click “continue”.

11. Load the sample tubes and consumables including the racks of black filter tips, deep well plate and the empty PCR elution plate as indicated in **Figure 2**. Follow the Deck layout as indicated in the diagram and **Table 3** to identify the carriers and positions to complete the loading process.

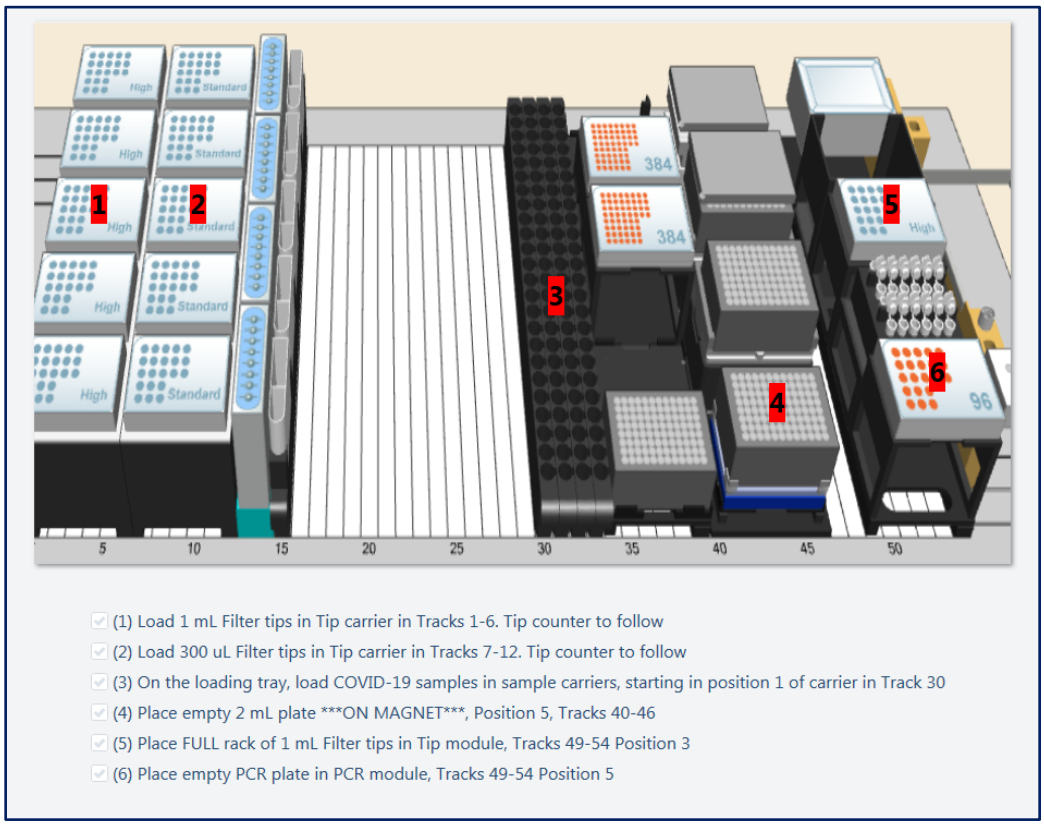


Figure 2. Deck layout diagram for consumables as shown in the GENFLEX™ Venus Software.

TABLE 3. Consumables to be Loaded

Loading Positions	Consumables	Cat. No.	Track #s
1	1 mL filter tips	Hamilton 235940	1-6
2	300 µL filter tips	Hamilton 235938	7-12
3	Sample tubes in carriers starting in slot 1	Sarstedt 72.703	30
4	2 mL plate “ON MAGNET”	Thomas Scientific 1162C08	40-46
5	Full rack of 1 mL filter tips in Tip module	Hamilton 235940	49-54
6	Empty PCR plate in PCR module	DN Biotech 5371009	49-54

12. Place the sample tubes with the extraction control and the specimens in the carrier and slide the carrier in through the tracks onto the loading platform. The barcode reading autoloader will pull the sample carrier into the deck of the instrument once the run commences. Until then, the sample carriers should remain on the loading platform. Place the deep well plate, which is referred to as the 2 ml plate "on magnet" as indicated in the table above and an empty PCR plate that serves as an elution plate on the PCR module as indicated on the deck layout and in the table above.
13. Click "Continue" after the consumable loading process is complete. The "continue" tab will only appear after the selection of the items in the checklist.
14. The next screen that appears will display the minimum number of 1 mL tips needed for the extraction for the inputted number of samples.
15. Click "Continue". The next window will display the layout of the 1 ml tips on deck. See **Figure 3**. Load the necessary number of tips until the plastic sleeve that holds each 96 tips snap into the tip carrier of the deck. Make sure that the number of tips loaded on deck at least meets or exceeds the number of tips necessary for the extraction.

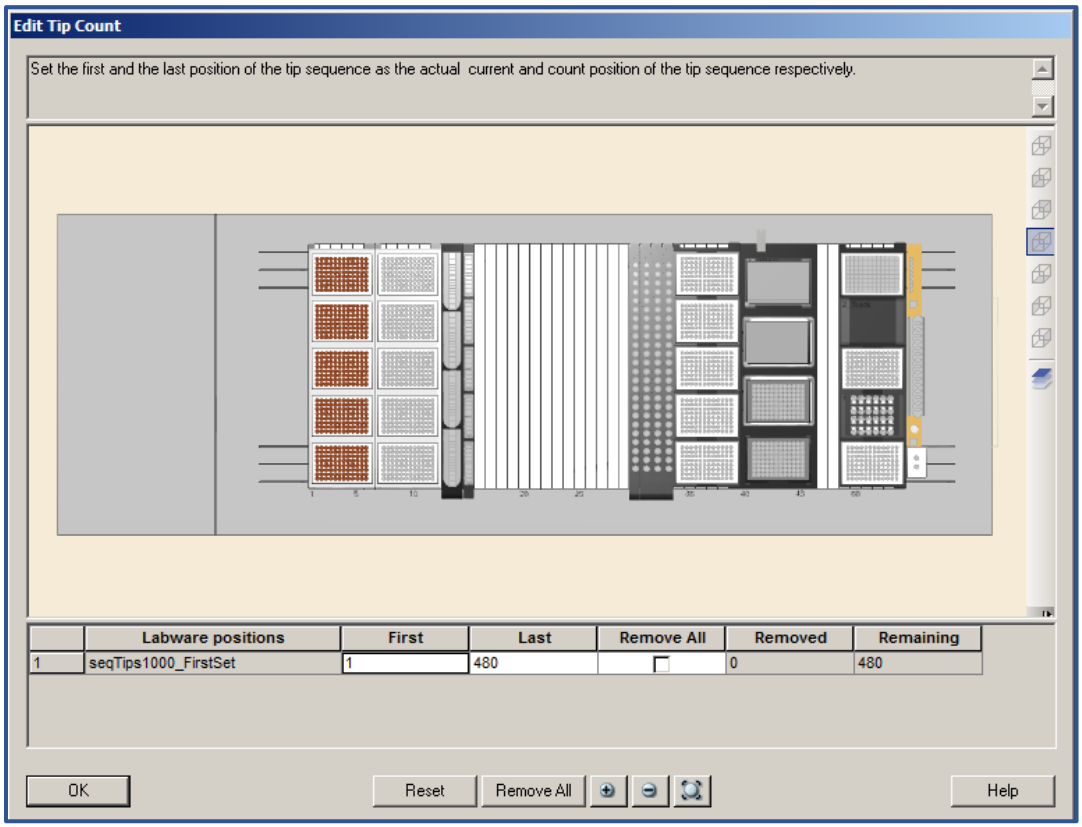


Figure 3. Location of loaded tips necessary for the run in GENFLEX™ Venus Software

16. In the interactive display, input actual location of 1 mL tips as loaded on the deck by clicking or dragging the tip positions. When selected, the loaded positions will be highlighted in brown.
17. Ensure that the physical arrangement of the 1 mL tips that was just loaded on the deck is identical to the arrangement of 1 mL tips highlighted in brown. If more tips needed to be added, zoom in on the display, and then select either individual tip locations, columns or the entire 96-well format by clicking or dragging the tip positions. Click "OK" after the positions of the tip sequence are updated in brown in the display matching the actual deck arrangement of the tips.
18. The following screens will display the number of 300 µL tips needed for the run as well the input window that requires the location of the tips to be updated will be displayed. Update the number of tips on the window displayed and then click "OK".
19. The extraction will start and several windows showing timers for incubation steps will appear during the run. No input from the user is necessary until the run finishes.
20. Once the run is completed, a window stating "AMPIPROBE is now complete" will be displayed.
21. Click "continue" to close the "Run Control" window of the software.
22. The samples are now ready for downstream applications. Keep the RNA elution plate sealed with the adhesive film at 4°C if PCR is being carried out within 2-3 hours, or at <-70°C for later use.

RT-PCR Master Mix (MMX) Preparation

Note: *The MMX preparation should be performed inside a PCR hood, and this hood must reside outside of the post-amplification area (preferably in the pre-amp room). All the reagents used in the MMX preparation are from the AMPIPROBE® SARS-CoV-2 Assay kit (RUO) (ENZ-GEN230-0100) and all controls are from the AMPIPROBE® SARS-CoV-2 Controls kit (RUO) (ENZ-GEN231-0004).*

1. Remove all of the RT-PCR reagents from AMPIPROBE® SARS-CoV-2 Assay (RUO) kit box and all PCR Controls from the AMPIPROBE® SARS-CoV-2 Controls (RUO) kit box. Thaw the reagents at room temperature while protected from light for 10-15 minutes, except the AMPIPROBE® RT-PCR Enzyme Mix. The RT-PCR Enzyme Mix from the kit should always remain on ice or in the -20°C freezer until needed for addition. All the other reagents, after being thawed, should be placed in an ice bucket or cool rack for holding during preparation.
2. The amount of MMX reagents required for the procedure can be calculated using the following table (**Table 4**).

TABLE 4. RT-PCR Master Mix (MMX)		
AMPIPROBE® MMX Reagents	**Volume per Sample or Control**	**Calculation for the Aliquot Procedure (n* + 40)**
RT-PCR Buffer	10 µL	10 x (n + 40) µL
SARS-Cov-2 Primer/Probe Mix	2 µL	2 x (n + 40) µL
RT-PCR Enzyme Mix	1 µL	1 x (n + 40) µL

***n** is the number of samples prepared, which includes the 3 AMPIPROBE® SARS-CoV-2 Controls and 1 Extraction Control (EC).

3. Once the amount of each reagent has been determined, use a 1.5 mL tube to prepare the MMX in. Vortex each tube in the kits (except for the Enzyme Mix) for three seconds and then briefly centrifuge them to remove any excess from the caps.
4. Add the MMX reagents in the order given in table above for the PCR setup. Instead of vortexing the RT-PCR Enzyme Mix, simply flick the tube gently three times and briefly centrifuge to remove excess from the cap, and then add the required volume to complete the MMX. Once the MMX is prepared, invert the tube ten times, briefly centrifuge, and then place on ice or at 2-8°C, protected from direct light until it is needed for the PCR setup.

   ***Note:** Prepared MMX may be kept for up to 20 minutes before loading and starting the Applied Biosystems QuantStudio® Real-time PCR system.*
5. Proceed to setting up for MMX aliquoting and sample RNA template addition by the GENFLEX™ platform.

GENFLEX™ Platform Setup for PCR Reactions

***Note:** The GENFLEX™ platform uses the Venus Software, Version 3.*

1. If PCR is being setup immediately following the extraction, do not power off the GENFLEX™ platform or close the Method Manager window on the desktop. If the instrument is powered off, turn the power switch on. The power switch (green switch) is located at the bottom-left corner of the instrument.
2. Double click on the “Method Manager” icon on the desktop of the computer. Click on “Run” next to COVID-19 icon, and select the “PCR” from the User Input Run Selection window.
3. In the next window displayed, select “96” for the 96-well format for the PCR, and click “continue”. Select the Sample Barcode file that has the sample information from ‘C:\Outputfiles\SampleBarcodes’. Select the Sample Barcode file with the correct time stamp and click Continue. A dialog box will appear to select if controls will be used in PCR. Select “Yes” and click “Continue”.

4. In the next window displayed, type in the number of samples for PCR setup as shown as on the third line of **Figure 4**. This would be similar to the format followed in the extraction. For an entire plate, 92 specimens can be processed along with one extraction control, which would be 93 samples. The number of PCR plates to prepare would be 1, the Master Mix volume would be 13 µL and the DNA template column, which is RNA, would be 12 µL out of the 60 µL total eluate from the extraction. In the next window, select the samples from the plate map. Note that the number of samples selected on the map must match the number of samples entered in the previous window. If the number of samples selected on map does not match the number of samples entered, an error message will prompt you to re-select samples on the map.

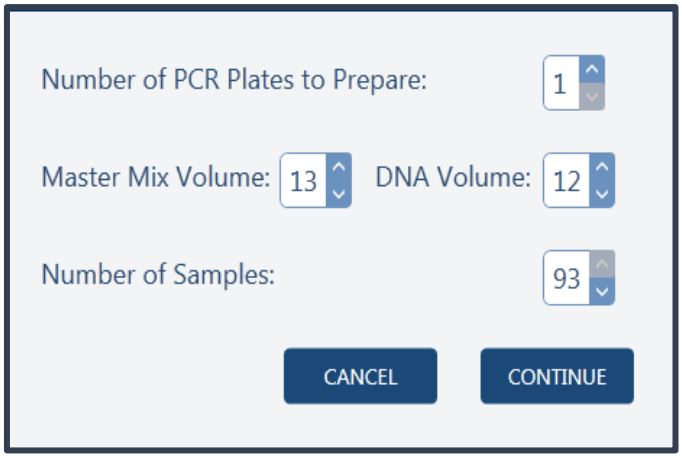


Figure 4. Input window displaying the number of samples for PCR.

5. In the next window displayed, choose the plate to which you want to add the controls (only one plate required for COVID assay, so the standard protocol is to have Plate 1 selected). Click continue and in the next window displayed, select multi-dispensing options. The standard protocol for COVID method is to have master mix multi-dispensed, and the RNA single-dispensed. The Deck Layout diagram showing locations of filter tips, PCR plate, RNA plate and the vials with standards and Master Mixes to be loaded is displayed next as shown in **Figure 5**. Load the filter tips, followed by the plates and the standards as shown in the diagram. After checking the list, click continue. A window to load and confirm 300 µL tip locations will appear similar to the diagram as shown in **Figure 3**. The multi-dispensing and the PCR setup will start and a window displaying “AMPIPROBE PCR setup complete” indicates the completion of the setup. Seal the plate with the adhesive film.

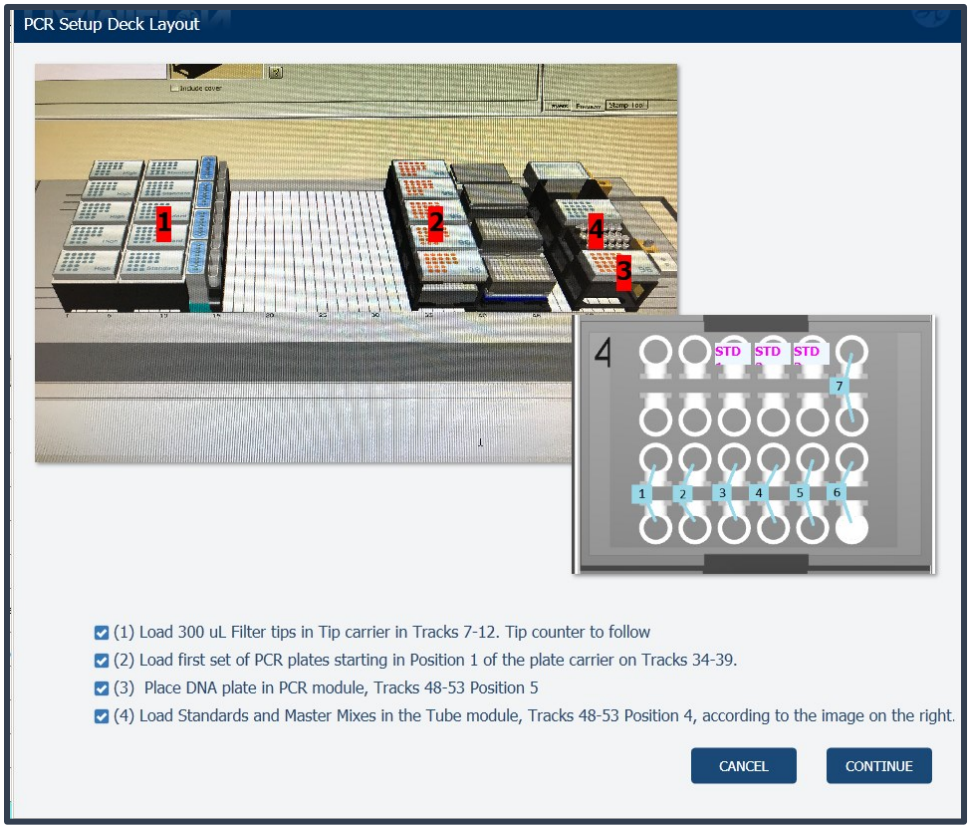


Figure 5. Diagram to load the tips, plates and the vials with locations specified.

6. Perform the RT-PCR using the procedure as described in the **RT-PCR Using the Applied Biosystems™ QuantStudio® 5 Real Time Instrument** section.

RNA EXTRACTION AND PREPARATION OF RT-PCR REACTIONS (Manual Method)

RNA EXTRACTION (Manual Method)

Manual extraction can be performed from a sample input volume of 350 µL using the AMPIXTRACT™ SARS-CoV-2 Extraction kit (RUO), ENZ-GEN225-0100.

Before You Begin

1. Determine the number of required reactions based on the number of samples to be processed and controls per plate.
2. Bring all samples to room temperature.
3. Prepare the AMPIXTRACT™ Wash Reagent and Lysis Reagent working solutions as described in **REAGENTS AND SAMPLE PREPARATION** section.
4. Thaw a tube(s) of **Stabilizer** reagent and place on ice.

5. Dilute the **Proteinase** reagent provided in the kit 30-fold in **PK Dilution Buffer** according to the following table:

Component	Volume per well*
Proteinase K	2 µL
PK dilution Buffer	58 µL
Total volume per well	**60 µL**

* Include 10% overage when making master mix for multiple wells.

6. Prepare **Complete Lysis Reagent** according to the following table:

Component	Volume per well*
Lysis Buffer	900 µL
Carrier RNA	10 µL
Stabilizer	14 µL
Total volume per well	**924 µL**

* Include 10% overage when making master mix for multiple wells

Process the Samples

Note: *If during aspiration of lysis supernatant, wash supernatant, or the eluate result in aspiration of the bead pellet, dispense the liquid back into its well, place the plate back on the plate magnet and carefully re-aspirate the liquid.*

1. Add 350 µL of samples to the wells of a Deep Well Plate (DWP).
2. Add 60 µL of the diluted Proteinase to each well to digest the samples. Incubate the plate for 10 minutes on a heater shaker set at 56°C and 500 rpm.
3. Lyse the samples by adding 924 µL Complete Lysis Reagent to each sample well. Incubate the plate for 15 minutes on the heater shaker set at 56°C and 500 rpm for efficient lysing.
4. Vortex the Magnetic Beads and add 20 µL to each sample well. Incubate the plate for 10 minutes on the heater shaker set at 56°C and 1100 rpm.
5. After incubation, place the plate on a plate magnet for a 96-well plate (e.g. Alpaqua # A000400) for 3 minutes. Aspirate the supernatant carefully without dislodging the bead pellet and discard the supernatant.
6. Remove the plate from the plate magnet, then add 950 µL of the Wash Buffer (pre-mixed with ethanol) to each sample well. Incubate the plate for 2 minutes on the heater shaker set at 56°C and 1300 rpm.
7. Wash and dry the beads.

    7.1 Place the plate on the plate magnet for 1 minute, aspirate the supernatant and discard.

7.2 Remove the plate from the plate magnet and repeat the wash step by adding 950 μL Wash Buffer (pre-mixed with ethanol), and then incubate for 2 minutes on the heater shaker set at 56°C and 1300 rpm.

7.3 Place the plate on the magnetic stand for 1 minute, and then aspirate and discard the supernatant. Completely remove washings to facilitate efficient drying of the beads in the next step.

7.4 Allow the beads to dry by placing the plate for 30 minutes on the heater shaker set at 56°C and 500 rpm. Make sure the beads are completely dry before proceeding to the elution step (step 7.5). To ensure efficient elution, beads should be completely dry.

8. Elute the RNA.

8.1 Add 60 μL of Elution Buffer to each well. Incubate the plate for 10 minutes at 56°C while shaking at 1400 rpm for elution of the nucleic acid.

8.2 Place the plate on the plate magnet for 1 minute. Remove and save the supernatant or eluate, which contains the SARS-CoV-2 RNA.

9. Store the eluate, which contains the extracted SARS-CoV-2 RNA, at <-70ºC for later use. Seal the plate immediately to prevent evaporation before transferring to <-70°C storage.

RT-PCR REACTION PREPARATION (Manual Method)

The procedure described below is for using the extracted sample RNA using an original sample input volume of 350 µL.

Before You Begin

1. To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area.
2. Do not use the same pipette for controls and samples. Always use aerosol barrier pipette tips.
3. The following controls provided in the AMPIPROBE® SARS-CoV-2 Assay Kit (RUO), ENZ-GEN231-0004, must be included for each run to accurately interpret results:
    - One Positive Control (PC) for PCR that contains SARS-CoV-2 targets N1 and N2, and human RNase P target.
    - One Negative Control (NC) for PCR that contains human RNase P target.
    - One No Template Control (NTC) that do not contain any targets.

    In addition to the above, an **Extraction Control (EC)** can also be included. The Extraction Control (EC) to use is a previously confirmed negative sample that is

taken through every extraction procedure. For additional information, refer to the **Quality Control** section.

Preparation of the Master Mix (MMX)

The Master Mix (MMX) is prepared using the reagents provided in the AMPIPROBE® SARS-CoV-2 Assay kit (RUO), ENZ-GEN230-0100.

1. The amount of MMX reagents required for the procedure can be calculated using the following table.

AMPIPROBE® MMX Reagents	Volume per Sample	Calculation for the Aliquot Procedure (n* + 3)
RT-PCR Buffer	10 µL	10 x (n + 3) µL
SARS-Cov-2 Primer/Probe Mix	2 µL	2 x (n + 3) µL
RT-PCR Enzyme Mix	1 µL	1 x (n + 3) µL

***n** is the number of samples prepared, which includes the 3 AMPIPROBE® SARS-CoV-2 Controls and 1 Extraction Control (EC).

2. Once the amount of each reagent has been determined, use a 1.5 mL tube to prepare the MMX in. Vortex each reagent from the kit (except for the RT-PCR Enzyme Mix) for three seconds and then briefly centrifuge to collect liquid at the bottom of the tubes.

3. Add the MMX reagents in the order given in table above for the PCR setup.

   ***Note:** DO NOT vortex the RT-PCR Enzyme Mix. Simply flick the tube gently three times, and then briefly centrifuge to collect liquid at the bottom of the tube.*

   Then add the required volume of the RT-PPCR Enzyme Mix to complete the MMX. Once the MMX is prepared, invert the tube ten times, briefly centrifuge, and then place on ice or at 2-8°C, protected from direct light, until it is needed for the PCR setup.

   ***Note:** Prepared MMX may be kept for up to 20 minutes on ice before loading and starting the Real-time PCR instrument.*

RT-PCR USING THE APPLIED BIOSYSTEMS™ QUANTSTUDIO® 5 REAL-TIME PCR INSTRUMENT

The procedure described below is for setting up and running RT-PCR using Applied Biosystems QuantStudio® 5 Real Time PCR instrument. For more information about the QuantStudio® 5 Real Time PCR instrument, refer to the instrument's manual. If using a different RT-PCR instrument, refer to the manufacturer's instrument manual, using the same cycling conditions.

Figure 6. Applied Biosystems QuantStudio® 5

Make sure the Applied Biosystems QuantStudio® 5 (shown in **Figure 6**) is powered on first, followed by the instrument's computer. The power switch for the instrument is located on the back of the instrument. Once both the instrument and computer have finished their start-ups, open the software "QuantStudio® Design and Analysis on the desktop. On the touch screen of the PCR instrument, go to "File" → "New Experiment" → "From template" → "COVID template 2". Then proceed as follows:

1. From the File menu, click "Import Plate Setup" and "Plate Import" file. Enter the file name with any relevant notes about the run (i.e. load name and date) in the "Experiment Properties" tab; and then click "Next".

   Verify that the temperature and cycling profile matches the settings given in **Table 5** below. Click "Next" and in the "Advanced Setup" tab, make sure the number of samples selected under the "Sample" tab matches the number of samples being processed. Make sure FAM, HEX and CY5 are shown and selected as "Targets". The green channel which is the green channel corresponds to SARS-CoV-2 N1 target, the yellow channel, which is HEX, corresponds to the RNase P target, and the red channel, which is the Cy5 corresponds to the SARS-CoV-2 N2 target.

TABLE 5. PCR Cycling and Temperature Conditions for the SARS-CoV-2 Assay				
Parameter	**Reverse Transcription**	**Taq Activation**	**Amplification/ Detection**	
Cycles	1	1	40	
Segment	1	1	1	2
Target [°C]	50°	95°	95°	58°
Hold [mm:ss]	30:00	02:00	00:03	00:30

2. Load the PCR plate containing the RNA and Master Mix into the PCR instrument. In order to load the plate, touch the eject instrument drawer icon (1) located on the top right as shown in **Figure 7**, and place the PCR plate with the reaction mixture into the drawer. Click “Start Run” in the Run tab.

Figure 7. Home Screen of Applied Biosystems QuantStudio® 5

3. Click on “Start Run” to begin the run.
4. Once the run is finished with the procedure, the raw data needs to be analyzed, it will be done to establish the result determinations for each sample.

DATA ANALYSIS

To perform data analysis and results interpretation, you must use the software that came with your RT-PCR instrument. The data analysis described below and the results interpretation in the following section are using Applied Biosystems QuantStudio® 5 Real-Time PCR instrument and software. For additional information, refer to the manufacturer's manual.

C(t) Determination

1. The amplification and detection performed on Applied Biosystems QuantStudio® 5 will be analyzed in the software to obtain the threshold crossing, C(t), for each channel.
2. For the PCR run, the green channel corresponds to SARS-CoV-2 N1 target, the yellow channel corresponds to RNase P, and the red channel corresponds to the SARS-CoV-2 N2 target.

RESULTS

The expected control results and interpretation are listed below. All controls must yield valid results for a test run to be valid. See **Table 6** below for controls interpretation.

TABLE 6. Control Sample Interpretation

Control	SARS-CoV-2 N1 C(t)	SARS-CoV-2 N1 C(t)	RNase P C(t)	Interpretation
Positive Control	< 40.0	< 40.0	<40.0	Valid result
Extraction Control	Negative, C(t) not detected	Negative	<40.0	Valid result
No Template Control (NTC)	Negative	Negative	Negative	Valid result

TROUBLESHOOTING

Category	Comments and Suggestions
General handling	During the run, if an error message appears, click “Repeat” and then click “Execute”. If error persists, click “Abort”.
Precipitation in reagents	Allow any precipitation to dissolve completely before loading the reagents into the instrument.
Magnetic particles are not completely resuspended.	Mix the bottle with the magnetic beads by vortexing before transferring the beads to the troughs. This ensures complete transfer of the beads to the troughs and better resuspension of the beads on the instrument.
Frozen samples are not mixed properly after thawing.	Mix the contents of the vial of the AMPIXTRACT™ Stabilizer with a manual pipette after thawing the product.
Degraded nucleic acids	All working surfaces should be cleaned with 10% bleach, followed by 70% ethanol solution before starting the extraction and PCR. Use RNase-free filtered pipette tips to prevent any introduction of RNases during the preparation of the Master Mix.
Incomplete sample lysis	Ensure that correct volume of ethanol is added to the AMPIXTRACT™ Lysis Reagent before loading onto the instrument.

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