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Protocol Number	TP-20-004	Protocol Title	Ubiquitome Limited SalivaDirect™ SARS-CoV-2 Test Modified for the Liberty16 System
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1 INTRODUCTION

- 1.1 Coronaviruses (CoV) are a large family of viruses that cause illness ranging from the common cold to more severe diseases such as Middle East Respiratory Syndrome (MERS-CoV) and Severe Acute Respiratory Syndrome (SARS-CoV). Coronavirus disease (COVID-19) is a new strain that was discovered in 2019.

The SalivaDirect™ SARS-CoV-2 test for the Liberty16 System is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test that uses saliva as the test matrix and two of the three Primer/Probe sets prescribed by the CDC EUA assay (EUA 200001).

The SARS-CoV-2 primer and probe set is designed to detect RNA from the SARS-CoV-2 in saliva specimens from patients as recommended for testing by public health authority guidelines. SARS-CoV-2 RNA is generally detectable in saliva 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. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities. If you are located outside this region check with your local regulatory authorities for the appropriate procedure with handling results.

Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

2 PRODUCT OVERVIEW AND TEST PRINCIPLE

- 2.1 The test is performed using the Liberty16 System (see **Table 1**), a mobile, battery operated, 16-well, real-time polymerase chain reaction (RT-PCR) device that connects via Bluetooth to the Liberty16 iPhone App for run set-up, real-time observations and post-run data analysis. The test is based on widely used nucleic acid amplification technology.

The test is a modification of the dualplex assay described as SalivaDirect™ by Vogels *et al.*, (SalivaDirect™: Simple and sensitive molecular diagnostic test for SARS-CoV-2 surveillance medRxiv 2020.08.03.20167791; doi: <https://doi.org/10.1101/2020.08.03.20167791>).

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The test has been modified to enable it to be run on the Liberty16 System which is a single fluorophore (FAM) device. The specific modifications are the use of FAM-labelled probes for both the viral (N1) and internal control (RNase P) targets which are run in separate wells on the Liberty16 real time PCR System. In all other respects the assay is identical to the published SalivaDirect™ protocol (Vogels, Doug E. Brackney, Chaney C Kalinich, Isabel M Ott, Nathan Grubaugh Anne Wyllie 2020. SalivaDirect™: RNA extraction-free SARS- CoV-2 diagnostics. [protocols.io https://dx.doi.org/10.17504/protocols.io.bkjgkujw](https://dx.doi.org/10.17504/protocols.io.bkjgkujw)

The test uses a primer/probe set targeting RNA from the SARS-CoV-2 nucleocapsid phosphoprotein gene (N1). An additional primer/probe set to detect the human RNase P gene (RP) is also included in the panel to serve as a sample adequacy and internal amplification control. The N1 and RP primer and probe sequences are described in the CDC EUA assay (previously FDA-authorized EUA200001) and are supplied at working concentration as a RUO kit by Integrated DNA Technologies (IDT, see Table 2 below). Note: only the N1 and RNase P primer/probe tubes are used from the kit. The RUO kit also includes the N2 primer/probe set which is not required for this assay.

- 2.2 A simple diagram of the test steps is presented in **Figure 1**. The test does not require specialized equipment for nucleic acid extraction, but instead uses a simple proteinase K treatment and heat inactivation step for sample preparation.

Figure 1: Liberty16 SARS-CoV-2 Saliva Test

Step 1. Saliva Collection

Simple collection receptacle
No preservation buffer



Step 2. Sample Preparation

Heat inactivation
with or w/o Proteinase K
treatment



Step 3. RT-PCR


Liberty16RealTimePCRDevice
Liberty16iphone/ipadApp



Step 4. Results - Liberty16 App

Review Controls
Report Results

Sample RNA is reverse transcribed to cDNA and subsequently amplified in the Liberty16 Real Time PCR Instrument. In 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 probe,

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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 through the Liberty16 iPhone/iPad App which is downloaded from the on-line Apple App store. The Liberty16 iPhone/iPad software interprets the data at the end of the reaction to provide qualitative test results for each channel.

The software application will display the result as Cq values on the iPhone/iPad once the cycle is completed. Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.


3 EQUIPMENT

3.1 **Table 1:** Instruments and software required to run the test

Component	Manufacturer	Model	Software/Version
Liberty16 Real Time PCR machine	Ubiquitome Limited	Liberty16	Firmware version 1.7.4
iPhone or iPad	Apple	Smartphone	iOS 13+ or latest
Liberty16 App for iPhone/iPad	Ubiquitome Limited. Downloaded from the Apple App Store		Version 1.8 (70)

3.2 **Table 2:** Reagents, Consumables and Equipment Required (But Not Provided)

Order the following Primer/Probe Kit	Manufacturer	Catalogue #
2019-nCoV RUO Kit, 500 rxn ^{Note1}	Integrated DNA Technologies (IDT)	10006713 (500 rxns)
Order one of the following Proteinases		
MagMAX Viral/Pathogen Proteinase K	Thermo Fisher	A42363 10 mL (4000 rxns)
Proteinase K, Molecular Biology Grade	New England Biolabs (NEB)	P8107S 2 mL (320 rxns)
Proteinase K	AmericanBio	AB00925 100 mg (800 rxns)
Order one of the following Master mixes		
TaqPath 1 Step RT qPCR Master Mix	Thermo Fisher	A15299 (1000 rxns) A15300 (5000 rxns)
Luna Universal Probe One-Step RT-qPCR Kit	New England Biolabs (NEB)	E3006S (200 rxns) E3006L (500 rxns) E3006X (1000 rxns) E3006E (2500 rxns)

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Reliance One-Step Multiplex RT-qPCR Supermix	BioRad	12010176 (200 rxns) 12010220 (1000 rxns) 12010221 (2000 rxns)
Order the following Control material		
Synthetic SARS-CoV-2 RNA Control 2	Twist BioScience	102024 (100 µL)
2019-nCoV N Positive Control	Integrated DNA Technologies	10006625 (250 µL)
Order PCR strip tubes & caps		
PCR Strip Tubes: White, Low profile (0.1 mL working volume) e.g. FrameStar Low, Break-A-Way, 96 well. PCR Plate –white tubes, black frame, or BioRad Strip-8, LO-PRO White	4titude® Ltd	4ti-1201
	BioRad	TLS0851
PCR Strip-tube optical caps appropriate for strip tubes eg. Framestar or BioRad tubes as described above	4titude® Ltd	4ti-0751 or 4ti-0783
	BioRad	TCS0803
General Lab Consumables Required		
DNAZap™ or equivalent	e.g. Ambion Cat	AM9890
RNAse Away™ or equivalent	e.g. Fisher Scientific	21-236-21
Molecular grade water, nuclease-free	Non-specific	Non-specific
10 % bleach (1:10 dilution of commercial 5.25-6.0 % hypochlorite bleach)	Non-specific	Non-specific
Aerosol barrier pipette tips (Nuclease-free)	Appropriate to micropipettes used.	Non-specific
1.5 mL microcentrifuge tubes (DNase/RNase free)	Non-specific	Non-specific
Disposable powder-free gloves and surgical gowns	Non-specific	
Equipment Required		
2 x 96-well -20°C cold blocks	Non-specific	Non-specific
Microcentrifuge	Non-specific	Non-specific
Micropipettes (2, 10, 200 and 1000 µl)	Non-specific	Non-specific
Multichannel micropipettes (5-50 µl)	Non-specific	Non-specific
Racks for 1.5 mL microcentrifuge tubes	Non-specific	Non-specific
Vortex mixer	Non-specific	Non-specific


Note 1: Use N1 and RNaseP tubes only.

4 SPECIMEN REQUIREMENTS

4.1 Acceptable Samples

4.1.1 Saliva that naturally pools into the mouth.

4.2 Sample Stability

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- 4.2.1 Specimens can be stored at room temperature for up to 6 hours.
- 4.2.2 Samples can be stored at 2–8°C for up to 7 days,
- 4.2.3 If a delay in sample processing is expected, store specimens at –80°C and thaw on ice for testing.

5 WARNINGS


This test **has not been FDA cleared or approved.**

6 SPECIMEN HANDLING AND PROCESSING

- 6.1 Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
- 6.2 Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- 6.3 Handle all specimens as if infectious using safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with SARS-CoV-2 (<https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html>).
- 6.4 Specimen processing should be performed in accordance with national biological safety regulations.
- 6.5 If infection with SARS-CoV-2 is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- 6.6 Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- 6.7 Use personal protective equipment such as (but not limited to) gloves, eye protection, and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.

7 WORK SPACE DECONTAMINATION

- 7.1 It is required to perform regular cleaning and decontamination of the work bench area as well as pipettes, racks, and small equipment.
- 7.2 Follow this decontamination process on a daily basis between runs, as well as before preparing reagents:
 - 7.2.1 Wipe area and tube holding racks with 2% bleach solution (2% active hypochlorite)
 - 7.2.2 Wipe with water
 - 7.2.3 Wipe with 70% ethanol
- 7.3 Follow this decontamination process once weekly or if environmental contamination is suspected:
 - 7.3.1 Soak bench surface area and tube holding rack in 2% bleach solution for 5-15 min
 - 7.3.2 Wipe pipettes outer surfaces with 2% bleach
 - 7.3.3 Rinse off surfaces with water
 - 7.3.4 Wipe with 70% ethanol

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7.4 **Important:** NEVER use bleach to clean the Liberty16 device. Clean the outer surface as follows:

- 7.4.1 Using a lint-free cloth slightly dampened with clean water, gently wipe the surfaces of the equipment. If a stronger cleaning agent is needed, use a lint-free cloth slightly dampened with 95% isopropyl alcohol.
- 7.4.2 If contamination inside the device is suspected, please contact Ubiquitome.

8 LABORATORY PRECAUTIONS

8.1 Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplification reactions. Incorrect results could occur if either the clinical specimen or the real-time PCR reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon).

- 8.1.1 Workflow in the laboratory should proceed in a unidirectional manner.
- 8.1.2 Maintain separate areas for assay setup and handling of nucleic acids.
- 8.1.3 Always check the expiration date prior to use. Do not use expired reagent.
- 8.1.4 Do not substitute or mix reagent from different kit lots or from other manufacturers.
- 8.1.5 Change aerosol barrier pipette tips between all manual liquid transfers.
- 8.1.6 During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the sample processing procedure. Proper aseptic technique should always be used when working with nucleic acids.
- 8.1.7 If available, use a PCR cabinet for mastermix preparations.
- 8.1.8 Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and sample processing .
- 8.1.9 Ensure all lab equipment has been validated and calibrated to manufacture instructions.
- 8.1.10 Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
- 8.1.11 Change gloves between samples and whenever contamination is suspected.
- 8.1.12 Keep reagent and reaction tubes capped or covered as much as possible.
- 8.1.13 Primers, probes (including aliquots), and enzyme master mix should be thawed and maintained on cold block at all times during preparation and use.
- 8.1.14 Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products at least daily such as 2% bleach, "DNAZap™" or "RNase AWAY®" to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
 - 8.1.14.1 Specific steps can be seen above in section 7.
- 8.1.15 RNA should be maintained on cold block or on ice during preparation and use to ensure stability.

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8.1.16 Dispose of unused kit reagents and all clinical waste such as human specimens according to local, state, and federal regulations.


9 REAGENT STORAGE, HANDLING, AND STABILITY

- 9.1 2019-nCoV CDC EUA Authorized qPCR Probe Assays are shipped at ambient temperature. Upon receipt, store as follows: –15 to –30°C in a constant temperature (non- frost-free) freezer through the expiration date provided on the Certificate of Analysis (CoA). Avoid repeated freezing and thawing.
- 9.2 Always check the expiration date prior to use. Do not use expired reagents.
- 9.3 Protect fluorogenic probes from light.
- 9.4 Primers, probes (including aliquots), and enzyme master mix should be thawed and kept on a cold block at all times during preparation and use.
- 9.5 Controls and aliquots of controls should be thawed and kept on ice at all times during preparation and use.

10 QUALITY CONTROL – as per SalivaDirect™ IFU

10.1 **Table 3:** Controls to Use with SalivaDirect™ SARS CoV-2 Test for Liberty16

Control Type	Description	Purpose	Frequency of Testing
Negative Template Control (NTC)	Nuclease-free water	To monitor for contamination of PCR reagents	Every batch of RT-PCR reactions set up and run at the same time
Positive Control	Twist Synthetic SARSCoV-2 RNA Control (Dilute to 100 copies/μL) or IDT 2019-nCoV N Positive Control (Dilute to 200 copies/μL)	To monitor the performance of RT-PCR reagents for N1.	Every batch of RT-PCR reactions set up
Human Specimen Control	Known negative saliva sample	To monitor the performance of RT-PCR reagents for RP.	Every batch of RT-PCR reactions set up
Internal Control	Primer/Probe set detecting RNaseP	Sample Adequacy and internal amplification control. Ensures sufficient quantity and quality of specimen was added.	In parallel with every sample

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11 PROCEDURE

11.1 Sample collection - As per SalivaDirect™ IFU (the following copied from Protocols io)

11.1.1 Saliva should be collected with the assistance of a healthcare worker or technician.

11.1.2 Before collection, clean hands using alcohol-based sanitizer or soap and water (no fragrances) and don appropriate PPE (at minimum, gloves and a mask).

11.1.3 Ensure all collection materials are labelled with the correct identifying information.

11.1.3.1 While preparing collection materials, direct the sample provider to begin pooling saliva in their mouth. Saliva production can be stimulated by thinking about food (favorite foods, upcoming meals, etc.) or about the saliva collection itself.

Note: This protocol is intended for the collection of the normal saliva that naturally pools into the mouth. No coughing or sniffing prior to sample collection is required.

11.1.3.2 Ideally, water should be avoided 10 minutes prior to collection. Other drinks, food, and nasal sprays should be avoided for half an hour before sample collection.

11.1.4 Remove the lid of the collection container and direct the sample provider to gently expel saliva into the container until at least 0.5 mL has been collected. Note: The total volume measured is to exclude any bubbles.

11.1.4.1 Once at least 0.5 mL has been collected, securely replace the lid of the collection container.

11.1.4.2 Sterilize the container surface with 70% ethanol or a disinfecting wipe and place the sample in a secondary container or an appropriately labeled biohazard bag.

11.1.4.3 Dispose of gloves and register the sample collection (including date and time).


11.1.5 Transfer the sample to the laboratory for sample processing. If the time between sample collection and the initial processing steps (aliquoting) is likely to exceed 6 hours, samples can be stored at 2-8°C for up to 7 days, or -80°C for long-term storage, then later thawed on ice for testing.

11.2 Extraction-free sample processing with proteinase K treatment

11.2.1 Add Proteinase K (see **table 4** for volume per sample) to designated 8-strip PCR tubes (low profile 100 µL working capacity). Include controls as specified above.

Table 4: Volume per sample

Vendor	Concentration	Volume
ThermoFisher Scientific MagMAX™ Viral/Pathogen Proteinase K	50 mg/mL	2.5 µL
New England Biolabs Proteinase K, Molecular Biology Grade	20 mg/mL	6.25 µL
AmericanBio Proteinase K Lyophilized	(add 50 mg per 1 mL of nuclease-free water)	2.5 µL

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This work should be completed under BSL-2 conditions, and samples potentially containing SARS-CoV-2 should only be handled in a biosafety cabinet. Please seek guidance from your local biosafety office on specific recommendations for working with samples which could contain SARS-CoV-2.

- 11.2.2 Vortex each saliva sample until homogeneous, and immediately transfer 50 µL saliva to each 8-strip PCR tube containing proteinase K.
- 11.2.3 Close the 8-strip tube lids tightly.
- 11.2.4 Briefly spin down the rack/tubes using a plate spinner or 8-strip tube microcentrifuge. Note: If no plate centrifuge or spinner is available, the plate can be gently tapped to get the samples at the bottom of each well.
- 11.2.5 Inactivate the proteinase K by heating samples for 5 minutes at 95°C on the Liberty16 PCR instrument or equivalent thermocycler. See **Appendix A** below for instructions on how to set-up the **Inactivation protocol** on the Liberty16.
- 11.2.6 Following incubation, remove samples from the device and briefly spin down the contents of the tubes using a plate spinner or 8-strip tube microcentrifuge.
- 11.2.7 Store samples at -80°C or proceed immediately to RT-qPCR testing.


11.3 PRIMER/PROBE PREPARATION

- 11.3.1 The CDC 2019-nCoV RUO kit includes all published assays for N1, N2 and RNaseP (RP) assays in individual tubes, which are premixed to the recommended working concentration for 500 reactions (see **Table 5** below). Primer/probe sets are delivered in IDTE (1X TE buffer) pH 7.5 and shipped at ambient temperature.
- 11.3.2 The SalivaDirect™ assay for Liberty16 **uses only the N1 and RNase P** components of the kit.
- 11.3.3 **Upon receipt, remove and set aside the N2 primer/probe tube. The N2 primer/probe set is NOT required for this assay.**
- 11.3.4 **Table 5:**

Tube	Components	Concentration as supplied (working concentration)	Concentration in final reaction
Primer/Probe Mix for either N1, N2 or RNaseP Assay	Forward Primer	6.7 µM	500 nM
	Reverse Primer	6.7 µM	500 nM
	Probe	1.7 µM	125 nM

- 11.3.5 Store primer/probe tubes at -15 to -30°C in a constant temperature (non-frost-free) freezer through to the expiration date provided on the Certificate of Analysis (CoA). Avoid repeated freezing and thawing.
 - Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.

11.4 Assay Set-up: Reaction Master Mix and Plate Set-up preparation

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- 11.4.1 In the pre-PCR clean room or PCR cabinet, place rRT-PCR buffer, enzyme and primer/probes on ice or cold- block. Keep cold during preparation and use.
- 11.4.2 Thaw reaction Mastermix prior to use.
- 11.4.3 Mix buffer, enzyme, and primer/probes by inversion 5 times.
- 11.4.4 Centrifuge buffer and primers/probe for 5 seconds to collect contents at the bottom of the tube and place the tube in a cold rack.
- 11.4.5 Label one 1.5 mL microcentrifuge tube for each primer/probe set (i.e., 2 tubes; one each for the N1 and RNaseP primer/probe sets).
- 11.4.6 Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, PC, HSC (if included in the RT-PCR run) and for pipetting error. Use the following guide to determine N:
- 11.4.6.1 If the number of samples (n) including controls equals 1-14, then $N=n+1$.
- 11.4.6.2 If the number of samples (n) including controls is 15 or greater, then $N=n+2$.
- 11.4.7 For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture ($N=\#$ of reactions) as follows in **Table 7**.

11.4.8 **Table 7**

Component	NEB Luna 2x	NEB Luna 4x	Bio-Rad Reliance	Thermo TaqPath
Master mix	N x 10 μ L	N x 5 μ L	N x 5 μ L	N x 5 μ L
RT	N x 1 μ L	-	-	-
Primer/Probe	N x 1.5 μ L	N x 1.5 μ L	N x 1.5 μ L	N x 1.5 μ L
Nuclease-free water	N x 2.5 μ L	N x 8.5 μ L	N x 8.5 μ L	N x 8.5 μ L


5 μ L Proteinase K- and/or heat-treated samples, standards, or controls will be added to each well individually in step 15, for a total 20 μ L reaction.

- 11.4.9 Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. Keep tubes on ice during this process. After addition of the reagents, mix reaction mixtures by pipetting up and down.
- 11.4.10 Centrifuge for 5 seconds to collect contents at the bottom of the tube and place the tube in a cold rack.
- 11.4.11 Set up new PCR strips (Low profile, white, 0.1 mL eg. 4titude® Ltd, cat. # 4ti-1201 profile, 8 strip tubes with flat optical strip caps).
- 11.4.12 Dispense 15 μ L of each master mix into the appropriate wells going across the row as shown below in **Figure 2** (using two 8-strip tubes, this is just a representation):

Figure 2:

	1	2	3	4	5	6	7	8
A	N1	N1	N1	N1	N1	N1	N1	N1
B	RP	RP	RP	RP	RP	RP	RP	RP

- 11.4.13 Prior to moving to the nucleic acid handling area, prepare the No Template control (NTC) reactions for column#7 in the assay preparation area.

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- 11.4.14 Pipette 5 µL of nuclease-free water into the NTC sample wells. (See **Figure 3**). One set of controls should be included in each batch of samples prepared and run at the same time.
- 11.4.15 Cover the entire reaction plate and move the plate to the specimen nucleic acid handling area.

Figure 3: Sample and control layout for run that includes positive and negative controls.


	1	2	3	4	5	6	7	8
A	S1	S2	S3	S4	S5	S6	NTC	PC
B	S1	S2	S3	S4	S5	S6	NTC	HSC

11.5 Nucleic Acid Template Addition

- 11.5.1 Bring the processed saliva samples and the PCR mastermix strip-tubes to a biosafety cabinet.

NOTE: Most institutions will require samples potentially containing full-length SARS-CoV-2 RNA to be handled in a biosafety cabinet. Please seek guidance from your local biosafety office on specific recommendations for working with samples which could contain full-length SARS-CoV-2 RNA. Gently vortex nucleic acid sample tubes for approximately 5 seconds.

- 11.5.2 Add 5 µL of extraction-free saliva sample to each designated well of the mastermix strip-tubes. Mix by pipetting, taking care to avoid introducing bubbles. Change tips after each addition.
- 11.5.3 Extraction-free saliva samples can be added using a multichannel pipette for high-throughput testing.
- 11.5.4 Add 5 µL of positive control (Twist synthetic SARS-CoV-2 RNA controls or IDT 2019-nCoV N Positive Control at 100 copies/µL) and human specimen control (HSC) to designated PCR wells. Mix by pipetting, taking care to avoid introducing bubbles. Place caps on tubes after sample addition.
- 11.5.5 If required, centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place tube in a cold rack.
- NOTE: DO NOT LABEL OR MARK THE TOP OF THE CAPS.
- 11.5.6 Once Sample Template and Assay Control addition has been completed, transfer strips to the Liberty16 Real Time PCR System. Align test strips so that **Sample A1 is the BOTTOM LEFT well, numbered "1" of the Liberty16 block (see Figure 15a)**.
- 11.5.7 See Appendix A below for instructions on how to set up and run the Liberty16 device.

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11.6 Extraction-free sample processing without proteinase K treatment

11.6.1 Perform the sample heat inactivation in a validated machine (e.g. thermocycler, dry heat block, convection oven etc.) at one of the validated settings: 95°C for 5 min; or 65°C for 15 min.

11.6.1.1 Alternatively, perform the heat inactivation in a separate 8-well PCR strip on the Liberty16 device: Transfer 20 µL of each sample (or pool – see 11.8.1) into a well (up to well 6), leave well 7 empty, and transfer human specimen control HSC (saliva of operator) into well 8. Tightly seal the strip with caps and heat-inactivate at 95°C for 5 min. Allow strip to cool down to ambient temperature. Remove strip from the Liberty-16 device. Spin down.

Figure 4: Layout for heat-treatment of saliva samples in PCR-strip.

Well	1	2	3	4	5	6	7	8
Sample	S1	S2	S3	S4	S5	S6	-	HSC

11.6.2 Follow the steps listed in 11.5 to set up the PCR reactions accordingly, using the heat-treated samples.

11.7 Data interpretation - As per SalivaDirect™ IFU

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

11.7.1 SARS-CoV-2 RT-PCR Test Controls – Positive, Negative, and Internal:

11.7.1.1 **Positive control:** The positive control should yield a “detected” result for the N1 target and “not detected” for the RNaseP control


11.7.1.2 **Human Specimen Control (HSC):** The HSC should yield a “not detected” result for the N1 target and “detected” for RNaseP

11.7.1.3 **Negative Template Control:** The NTC should yield a “not detected” result for both the N1 and RNaseP targets

11.7.1.4 **Internal Control:** Detection of RNaseP at PCR cycle threshold (Cq) <35 indicates that saliva of sufficient quantity and quality were tested. Detection of RNaseP is required to report a negative SARS-CoV-2 result.

Subsequently, the tested samples will be interpreted according to the following table for individual (not pooled) samples:

Result	Cq Value of N1	Cq Value of RP
Positive	< 40	Any Value
Negative	≥ 40 or na	< 35
*Invalid	≥ 40 or na	≥ 35

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***Note:** Invalid test results will be repeated by retesting the primary specimen from the beginning of the protocol. Results from retested samples will follow the same interpretation as listed in the table above.

11.8 Pool Sample Testing

11.8.1 A total of 50 µL of each individual saliva sample is suggested to pool together (for a maximum of 5 samples) then run through the standard SalivaDirect™ protocol.

NOTE: The SalivaDirect™ pooled protocol resulted in a positive percent agreement of 86.4% (19/22, 95% CI: 66.67%, 95.25%) compared to the individual testing results using the standard SalivaDirect™ protocol. Overall, a theoretical Cq shift of Log₂(n) can be estimated for most RT-PCR tests due to the dilution of positive samples when pooled with negative samples. This means that for pools of n=5, a Cq shift of 2.3 would be expected.

11.8.2 Pooled Sample Results and Reflex Testing

The interpretation of results for pooled SalivaDirect™ testing is the same for all thermocyclers used. Results of pooled testing should be interpreted according to the following (and summarized in the table below):

Negative Result: If samples were pooled and the SARS-CoV-2 N1 target is not detected at all (not detected, ND; NaN; Undetected; ≥45.0; etc.), then all samples in that pool should be reported as Negative. Negative results from pooled sample testing should not be treated as definitive. If the patient’s clinical signs and symptoms are inconsistent with a negative result and if results are necessary for patient management, then the patient should be considered for individual testing. The utilization of sample pooling should be indicated for any specimens with reported negative results. RP must be detected with Cq <30.

Not Negative Result (i.e., positive or invalid): If samples were pooled and determined not negative through generating a Cq value of any value for N1 (<45.0) or returns an invalid result (poor or no RP detection), then all samples in that pool should be tested individually by the laboratory’s standard SalivaDirect™ protocol prior to result reporting. Only the results of the individually tested samples (as interpreted depending on the thermocycler used; tables above) should be reported.

Figure 4: Pooled SalivaDirect™ results, interpretation and action.

Cq Value		Interpretation	Action
N1	RP		
≥ 45 or na	< 30	Negative	Report all samples as Negative
≥ 45 or na	≥ 30	Not negative: Invalid	Reflex test all samples individually
Any value	< 45	Not negative: Positive	Reflex test all samples individually




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APPENDIX A: Liberty16 System – Instructions for Use

1. SETTING UP A PROTOCOL ON THE LIBERTY16 DEVICE AND RUNNING THE TEST

- 1.1. **Download the Liberty16 Application onto your iPhone or iPad** (Required if not already installed). Note: you will need to establish an Apple ID to download the App. The Liberty16 device is operated from the iPhone/iPad Application via a Bluetooth connection. If a copy of the App is already installed, go to the next section “Create a PCR Run Protocol”
 - 1.1.1. Go to the App Store on your device and search for Ubiquitome. You should see the Ubiquitome butterfly symbol.
 - 1.1.2. Download the App onto each iPhone/iPad that will be used with your Liberty 16 device(s).
 - 1.1.3. Turn on the Liberty16 instrument with a single press of the small “Power” button on the rear of the instrument. The Ubiquitome butterfly icon will light up red.
 - 1.1.4. Ensure Bluetooth is activated on the iPad/iPhone. Open the Liberty 16 Application and select the Home screen from the menu at the bottom of the screen. The program will automatically search for and detect an active device.
 - 1.1.5. If you have never connected to this device before, a dialog pop-up will appear on the main screen asking for a pin before pairing (**Figure 7**).
 - 1.1.5.1. Use the last SIX digits of the serial number as the pin number
 - 1.1.5.1.1. The serial number can be found on the white sticker on the bottom of the Liberty16
 - 1.1.5.2. If you have an older device that does not have a serial number, just press ‘Pair’ without inputting any pin number.
 - 1.1.6. The Ubiquitome butterfly icon on the front of the instrument should change from red to green once it has paired with the App.
 - 1.1.7. The App will automatically register the firmware version of software on the paired device (fw 1.7.4). If a firmware update is required, a dialog pop-up will appear on the main screen (**Figure 4**). If no update is required, proceed directly to setting up and running a protocol (see next section below).
 - 1.1.8. If an upgrade is required, you will be given the option of backing up your data. Note: Updating the firmware does NOT delete protocols or past runs. This is simply for added safety. If you’d like to back up your run data before updating the firmware, select “Back Up My Data”. You will be taken to the Runs screen. Swipe right to left on any run and email it or upload it to iCloud. Repeat for all runs you wish to save (**Figure 5**).
 - 1.1.9. Once you have backed up all desired runs, return to the Home page.
 - 1.1.10. The same dialog box will pop up. Select “Update my firmware”
 - 1.1.11. A new screen will appear showing the update progress on your device.

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1.1.12. When the download is complete, the firmware will be installed, and a pop-up window will appear instructing you to restart the Liberty16 and the App (**Figure 6**).

1.1.13. Shut down both the Liberty16 and the App. Restart the Liberty16 then re-open the App. The new firmware version will be displayed on the home screen and you are now ready to set up and run a protocol.

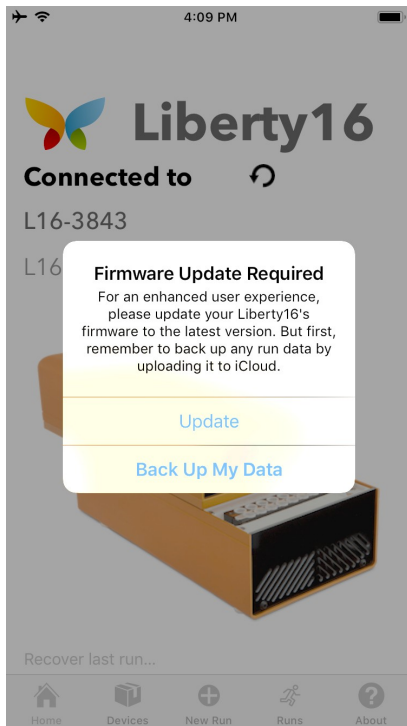


Figure 4: Pop-up window

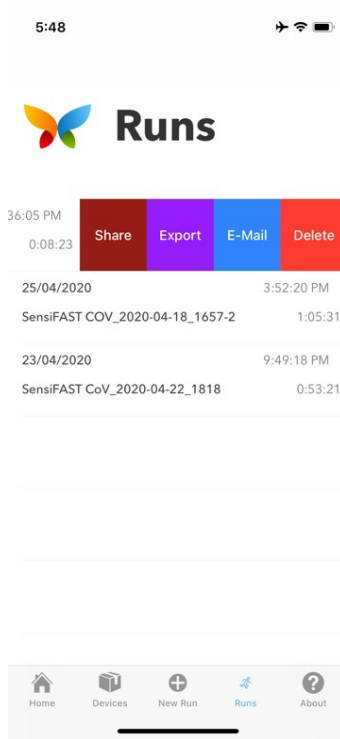


Figure 5: File options

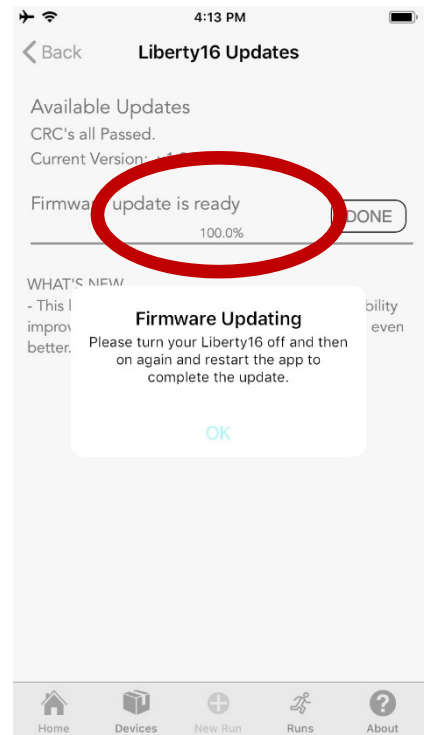


Figure 6: Pop-up message after download complete.


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Figure 7: Liberty16 Home Screen with pairing request and after pairing is complete – Device name is shown. To change connected device, tap on My Devices at the bottom of the screen.

- 1.2. **Creating a PCR Run Protocol for the Liberty16 Real-time PCR Instrument** (Required if no template exists) If the template already exists on the instrument, please proceed to the **Running a Test** section. Otherwise, proceed as follows.
 - 1.2.1. Turn on the Liberty 16 with a single press of the small “Power” button on the rear of the instrument. The Ubiquitome butterfly icon on the front of the instrument should light up red and transition to green when connected to the Ubiquitome Liberty16 iPhone/iPad Application.
 - 1.2.2. Open the Liberty16 Application on your iPhone/iPad and select the **Home** screen from the menu at the bottom of the screen. The program will automatically search for and detect an active device.
 - 1.2.2.1. If multiple devices are in range, we recommend to turn off all but one and connect them one at a time.
 - 1.2.3. If you have never connected to this device before, a dialog pop-up will appear on the main screen asking for a pin before pairing (**Figure 7**)
 - 1.2.3.1. Use the last SIX digits of the serial number as the pin number.
 - 1.2.3.2. The serial number is a nine-digit number that can be found on the white sticker on the bottom of the Liberty16.
 - 1.2.4. To create a new PCR run protocol, select **New Run** from the menu at the bottom of the screen (**Figure 8**).
 - 1.2.5. Click the (+) sign at the top right-hand side of the New Run window and enter the protocol name in the pop-up window. Press OK. (**Figure 9**)



Figure 8: New Run Screen
Click + to create a new Run Protocol

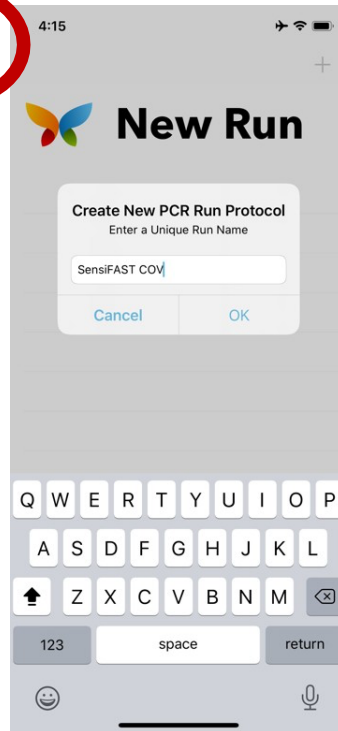


Figure 9: Enter Protocol Name.
Click OK.

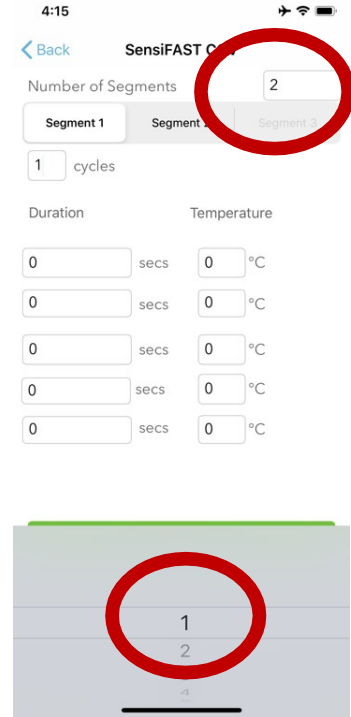



Figure 10: Click number of Segments at top of screen.
Select from menu at bottom.

1.2.6. The protocol set-up screen will appear. Protocols are written as sequential segments, each segment representing a different number of cycles. Enter the **Number of Segments** required (**Figure 10**). Note this will differ for the Extraction-free sample processing (Section B) and the SalivaDirect™ reaction protocols as described in the examples below.

Example 1: Incubation Protocol: for Proteinase K incubation step described in Section B: Extraction-free sample processing. This protocol comprises 1 segment and the following time temperature regime:

Incubation Protocol for Extraction Free Sample Processing			
Segment	Process	Cycles	Time/Temperature
1	Sample Incubation	1	5 min, 95 °C

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Example 2: RT-PCR Protocol: for Liberty16 SalivaDirect™ assay. This protocol comprises 2 segments and the following time/temperature regime.

SalivaDirect™ RT-PCR protocol – <i>Note Protocol shown in pictures below is for illustration only</i>			
Segment	Process	Cycles	Time/Temperature
1	RT Activation	1	10 min, 52 °C 2 min, 95 °C
2	PCR cycling	44	10sec, 95°C 30sec, 55°C

^a Thermal cycling time/temperature protocols may differ depending on the mastermix/polymerase combinations used.

1.2.7. Select the **Segment 1** tab.


- 1.2.7.1. Enter the number of **Cycles** using the selection menu at the bottom of the page [1 for SalivaDirect™ RT-PCR and Sample prep protocols] (**Figure 11**).
- 1.2.7.2. Enter the **Duration** for the first step by first clicking on the duration window, then selecting the time using the selection menu at the bottom of the page. The duration will appear in seconds in the duration cell [10 minutes for SalivaDirect™ RT-PCR protocol].
- 1.2.7.3. Enter the **Temperature** for the first step using the same process as above [52 °C for SalivaDirect™ RT-PCR protocol].
- 1.2.7.4. Repeat b – c for other steps in the segment.
- 1.2.7.5. If there are no further segments, skip ahead to **Step 1.2.9**.

1.2.8. Select the **Segment 2** tab.

- 1.2.8.1. Enter number of **Cycles** (e.g. 44 for SalivaDirect™ RT-PCR protocol above)
- 1.2.8.2. Enter Duration/Temperature conditions for each step using the same process as described for Segment 1.
- 1.2.8.3. **IMPORTANT.** Turn on the **Sample** switch adjacent to the step of the process for which fluorescence data is to be recorded. For the SalivaDirect™ protocol this should be the PCR extension step (30 sec, 55 °C). See **Figure 12**. *The sample switch must be selected for the device to collect fluorescence data during the run. This is the first thing to check if your run appears to have no data.*

1.2.9. Exit the Duration/Temperature selection menu by touching on a blank area of the screen. You should now be able to see the green **Save PCR Protocol** option. Click on this to save the protocol (**Figure 13**).

1.2.10. The new protocol will now appear in the **New Run** screen. If you need to edit the protocol, swipe right to left on the protocol name and select **Edit**.

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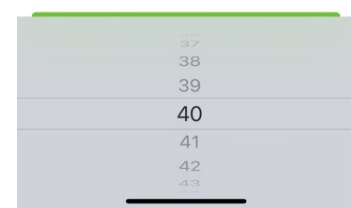
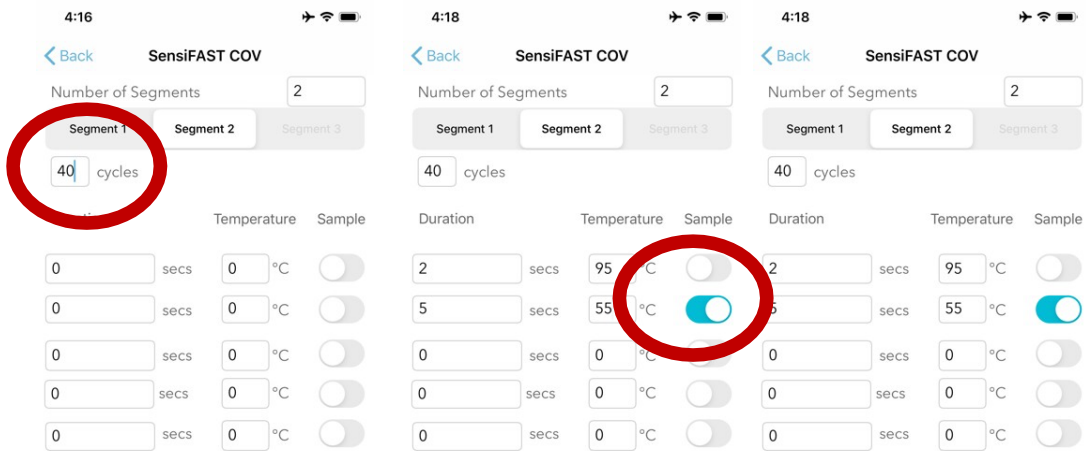


Figure 11: Segment 2 screen. Cycle number for this Protocol is set at 42.

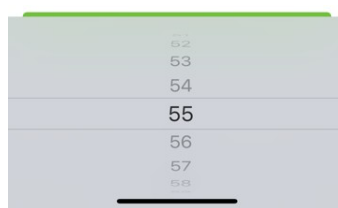


Figure 12: Select Segment step for data collection.

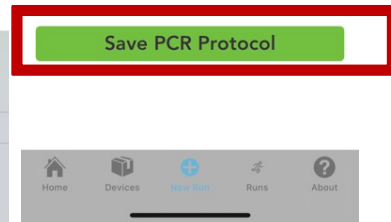



Figure 13: Save PCR Protocol

1.3. Running a Protocol on the Liberty16

- 1.3.1. **Turn on** instrument and **open the Liberty16 Application** on the iPhone/iPad (if not done already).
- 1.3.2. If starting up, confirm that the app is paired to the correct Liberty16 instrument by going to the **Devices** menu at the bottom of the page. The ID of the paired device will appear in the **My Devices** window.
- 1.3.3. Click on the **New Run** and select the desired protocol from the list of saved protocols (**Figure 14**).
- 1.3.4. Click on the green **Name Wells** tab to bring up the sample identifier panel (**Figure 15**). The 16 wells are listed. Wells 1 – 8 and 9 - 16 represent the wells from left to right on the lower and upper strips respectively (**Figure 15A**). Enter sample/patient identifiers for each well. Click on the (x) to clear existing name or simply delete existing data and enter new name.
- 1.3.5. Click on the green **Start PCR Run** to commence the run. The PCR run screen will appear showing the temperature and fluorescence profiles in real time throughout the run (**Figure 16**). The window can be closed. Once the run is completed the instrument will automatically shut down after approximately 20 minutes of inactivity. The data is saved

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in real time to the iPad/iPhone. Note: Only temperature data will be recorded for the Sample Incubation protocol described above.

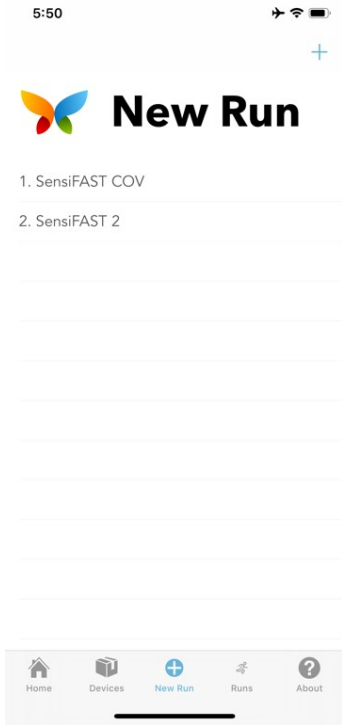


Figure 14: Select from saved Protocols.

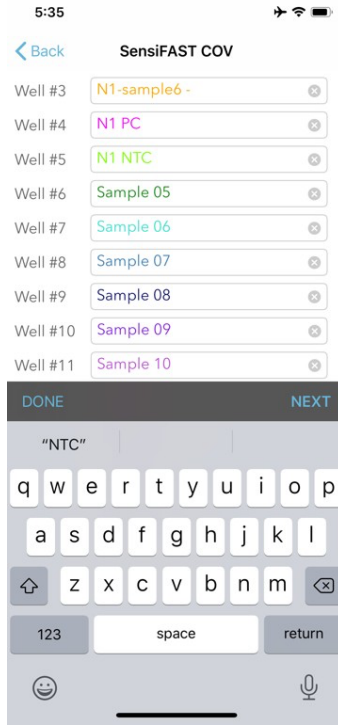


Figure 15: Enter sample details.

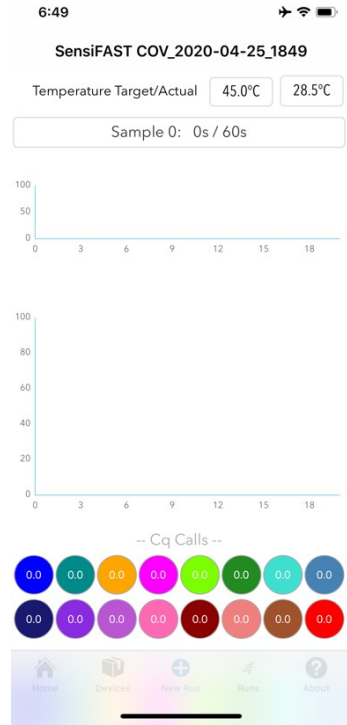



Figure 16: PCR run screen showing real time data collection.

Figure 15a: Layout of the Liberty16 plate. Well numbers correspond to Sample numbers in the Sample



name window.

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1.4. Retrieving and viewing data from the Liberty16

1.4.1. Open the Liberty16 App on the iPhone/iPad

1.4.2. Select the **Runs** tab from the menu at the bottom of the screen (**Figure 17**). The latest run will appear at the top of the screen. The filename will include the Protocol name, date and time of run completion.

1.4.3. Select the Run to see the PCR screen (**Figure 18-20**). This will show the PCR plots for each well. Click on any of the colored buttons at the bottom of the screen to select an individual well. These buttons can be toggled on or off, enabling comparison between selected samples.

1.4.4. Click on the **Cq Calls** tab at the top of the screen to see the Cq values for each sample.

1.4.5. The **Protocol** tab at the top of the screen shows the protocol used.

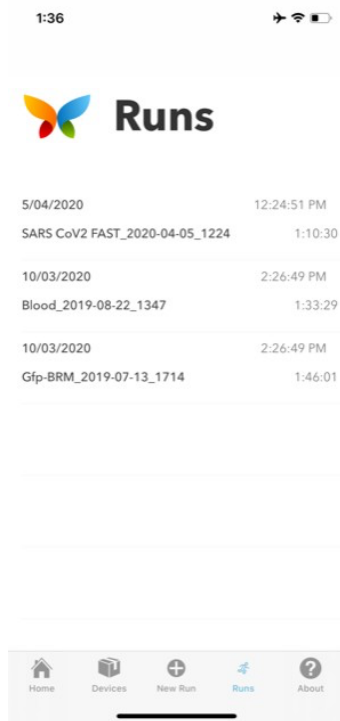


Figure 17: Runs screen lists results reports.

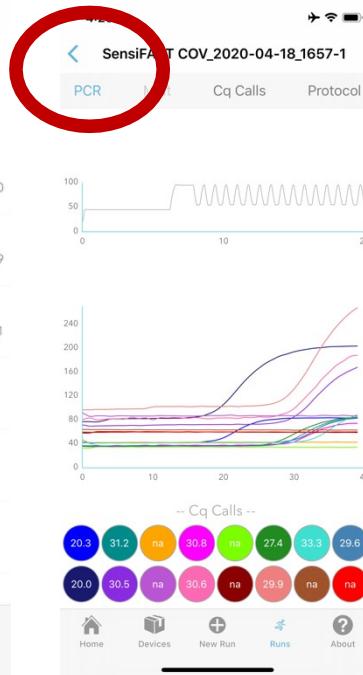


Figure 18: PCR report

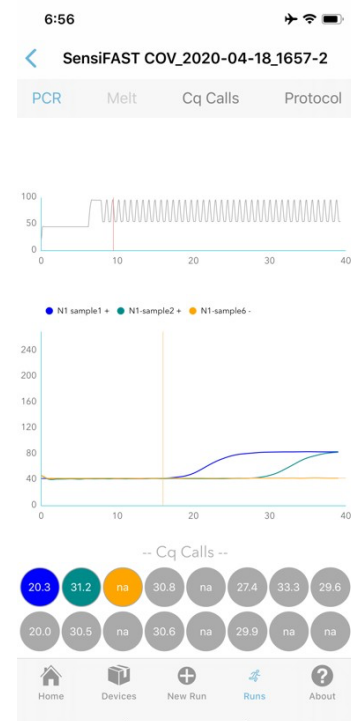


Figure 19: PCR report for selected samples.

1.5. Exporting data from the Liberty16

1.5.1. Select the **Runs** menu from the bottom of the screen.

1.5.2. **Swipe from right to left** on the run of choice to bring up a selection of data retrieval options (**Figure 20**).

1.5.2.1. **Delete** - removes the run file

1.5.2.2. **Export** - exports the log file (including Cq data) in simple text to cloud-based file

1.5.2.3. **Share** – enables the log file (including Cq data) to be shared via text

message

1.5.2.4. **E-Mail** – attaches the full log and data file to an email. This option enables the files to be opened in the recipients Ubiquitome Liberty16 App providing full functionality.

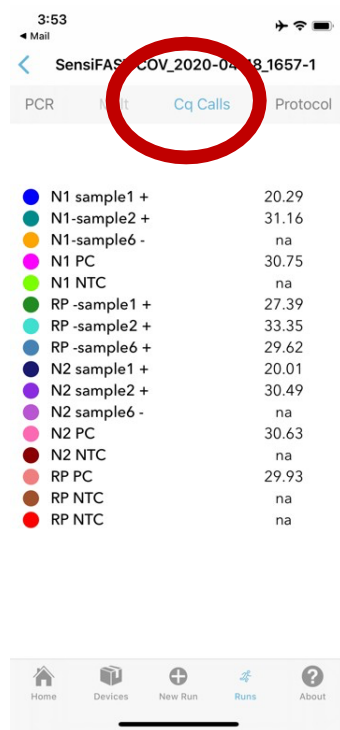


Figure 20: Cq Data screen for Selected wells.

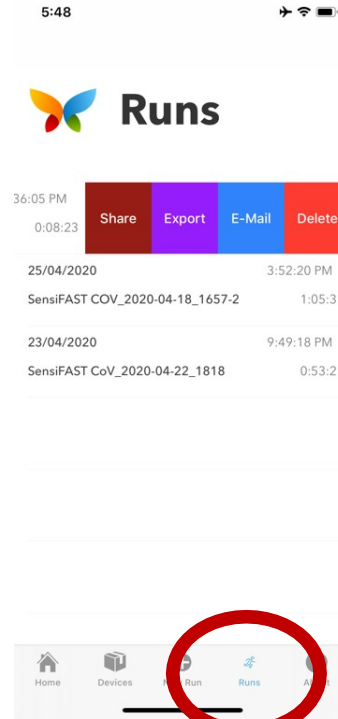



Figure 21: Data export from Runs Screen. Swipe Right to Left on run Name to bring up options.

1.6. Viewing data in qualitative mode

1.6.1. Cq data for each completed run can be viewed as either a quantitative Cq value (default) or as a qualitative score (Qualitative Mode). To select qualitative mode:

- 1.6.1.1. Select the **“About”** menu from the bottom of the home screen.
- 1.6.1.2. Turn on Qualitative Mode switch (**Figure 22**).
- 1.6.1.3. Open the run data file of interest and select Cq. The data values will be displayed as a score ranging from Negative to Very Strong Positive (**Figure 23**). To return to quantitative values, open the **About** menu, switch off qualitative mode and re- open the data file.

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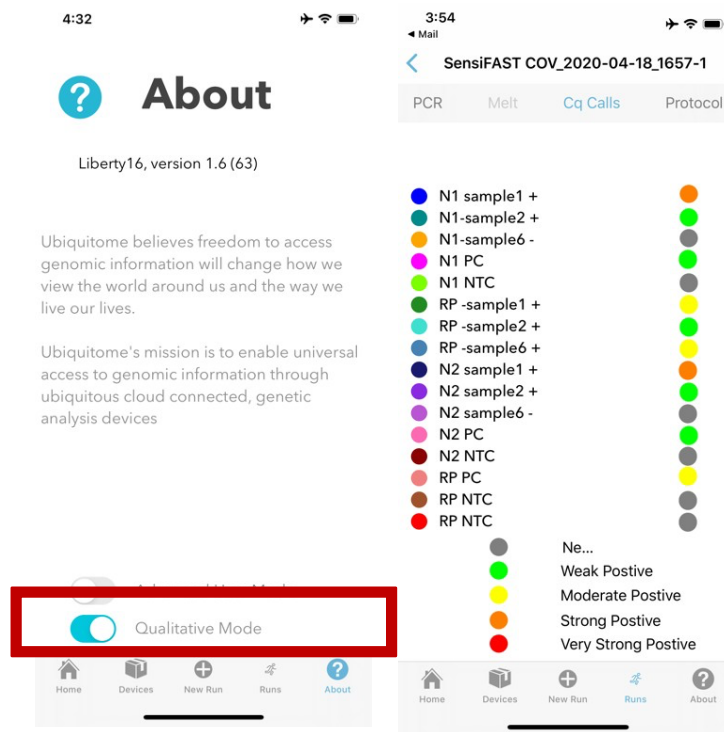


Figure 22: Toggle switch turns On Qualitative mode

Figure 23: Cq data presented in Qualitative mode.

2. DATA ANALYSIS

- 2.1. Review the amplification plots for any “false” calls.
- 2.2. Positive samples have amplification plots with exponential growth.
- 2.3. Negative samples have no amplification plots.

3. INTERPRETATION OF RESULTS

- 3.1. For more details on interpretation, see section 11.7 in the main document.

4. REFERENCES

- 4.1. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel SOP
- 4.2. EMERGENCY USE AUTHORIZATION (EUA) SUMMARY: SARS-CoV-2 RT-PCR Assay. Yale School of Public Health, Department of Epidemiology of Microbial Diseases. Oct 2021. Available at: <https://www.fda.gov/media/141192/download>