1.0 Objective

The UC Davis High Throughput (UCD HTP) COVID-19 saliva test is intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in saliva samples. This method describes the procedure to prepare, analyze, and evaluate saliva samples for SARS-CoV-2 with real-time reverse transcription PCR (rRT-PCR) on a high throughput instrument.

2.0 Reagents and Materials

The following equipment and reagents are required. Equivalent equipment or reagents may be substituted.

2.1. LGC IntelliQube High-throughput qPCR system

2.2. FluidX IntelliXcap 96-tube Decapper or equivalent

2.3. FluidX Perception™ HD Plate Scanner with IntelliCode software

2.4. Integra 25 mL trough insert; PN#4311, 4312 or equivalent

2.5. Integra ViaFlow 12-channel automated pipettes with Assist Plus robotic system, a Viaflo-96, or equivalent

2.6. Analytical balance

2.7. Handheld 2D barcode scanner

2.8. Micropipettes 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL

2.9. Graduated cylinder and serological pipettes

2.10. 96-well 0.8 mL storage plate; LGC #KBS-7001-131

2.11. 384-well V-bottom plate; Greiner Bio-One #781280

2.12. qPCR master mix:

LGC RapiDxFire qPCR 5X Master Mix GF 10 mL; LGC #30050-2, and EpiScript RNase H Reverse Transcriptase 25,000 units; LGC #ERT12925K

or

UltraPlex 1-Step ToughMix (4X); Quantabio #95166 or equivalent

2.13. 2019-nCov CDC Probe and Primer Kit for SAR-CoV-2; LGC #KIT-nCoV-PP1-1000 or equivalent.

2.14. BEI gamma irradiated, SARS-CoV-2 virus; BEI Resources NR-52287

2.15. SuperROX® Reference Dye; Biosearch Technologies #SR-1000

2.16. 10x TBE buffer (Tris, Boric Acid, EDTA); Bio-RAD #1610733 or equivalent

2.17. TE buffer (10 mM Tris, 1 M EDTA, pH 8.0); Invitrogen #AM9849 or equivalent

2.18. Low-EDTA TE buffer (10 mM Tris, 0.1 M EDTA, pH 8.0); Teknova #T0223 or equivalent.

2.19. Papain (powder, purified); Spectrum Chemical #PA125.

2.20. RiboGuard RNase Inhibitor (40x); Biosearch Technologies #RG90910K or equivalent.

2.21. Water, RNase free, Molecular Biology Grade; Corning #46-000-CM or equivalent.

2.22. Computer, Windows 10, with Internet access and USB3 port.

3.0 Preparation of Reagents, Standards, and Sample Solutions
Alternate volumes may be used, as long as the concentration remains the same.

3.1. 1X TBE Solution
Add 5 mL 10X TBE in a graduated cylinder, then fill to the 50 mL mark with RNase-free water.

3.2. 5% Papain Solution
Weigh out 2.25 g Papain and transfer to a 50 mL conical tube. Fill to 35 mL with 10x TBE Solution. Allow Papain to dissolve, then fill to 45 mL. This solution is stable for 7 days when stored at 4°C.

3.3. 80x 2019-nCoV Primer and Probe
The lyophilized 2019-nCoV Primer and Probe come in pre-aliquoted microfuge tubes. Allow these tubes to reach ambient temperature, then briefly centrifuge for 10 seconds using a microcentrifuge. Resuspend each Primer and Probe mix (N1, N2 and RNase P) by adding 250 µl 1X Low-EDTA TE Buffer and vortexing briefly to dissolve. Centrifuge again to collect all liquid at the bottom of the tube. Store at 4°C, protected from light, for up to a month.

3.4. N2 Spiked Probe Mix
Transfer 51.3 µL of the N2 Probe Mix to a clean microtube and add 7 µL 200 µM N2F Primer and 7 µL 200 µM N2R Primer. Pipette up and down to mix. Store at 4°C, protected from light, for up to a month.

3.5. Assay Mix
Make one large mix of PCR reagents for all three assays using RapiDxFire qPCR 5X Master Mix GF, EpiScript RNase H Reverse Transcriptase, SuperROX, and RNase-free water in a ratio of 0.400 : 0.050 : 0.010 : 0.515. Mix to combine, then divide into thirds. Add the appropriate Primer and Probe mix at the following ratio: N1 (0.025), Spiked N2 (0.030), and RNase P (0.025). Prepare fresh daily and shield from light
    or
Make one large mix of PCR reagents for all three assays using QuantaBio UltraPlex Master Mix (4X), SuperRox (15uM), and water in a 0.500 : 0.010 : 0.465 ratio. For example, 232.2 µL of MasterMix, 4.6 µL SuperRox, and 2.15.9 µL water. Mix to combine then divide into thirds. Prepare fresh daily.

3.6. Control Samples

COVP1 control:	BEI gamma irradiated virus controls diluted in TE buffer/1x Riboguard (or equivalent) to 10,000 copies per microliter. Then added to negative human saliva samples to a final concentration of 250 copies per µL.
NTC (no template control):	1x TBE buffer.

4.0 <u>Receiving Procedure</u>

4.1. Samples will arrive in 1 mL screw-top tubes in a 96-tube format.

4.2. Place sample rack(s) in an 80-85°C water bath for 15 min. Place up to four racks in the water bath at a time. If we receive more than four racks, keep the remaining racks in the plastic tub labeled "Non-deactivated saliva" until they are able to be processed. Refer to TD-0006.

4.3. After 15 min. remove sample rack(s) from the water bath. Place paper towels or other absorbent material between the sample tube caps and sample rack lid, and shake to remove excess fluid from the sample tube caps. Then remove the rack lid and allow to cool to room temperature and air dry.

4.4. Sample tubes are then scanned with a *FluidX Perception HD plate scanner*, and sample information linked to a specific rack and position as outlined in TD-0002.

4.5. After the sample tubes have been linked to their respective rack in the LIMS software in TD-0001, transfer the entire rack to the *FluidX IntelliXcap*, remove the rack safety lid, and remove the caps from the sample tubes as outlined in TD-0003.

4.6. After decapping, move the sample rack to the *Integra Assist Plus* in *Position B*.

4.7. Load a rack of 300 µL pipette tips onto the *Assist Plus Position 1* and the *FluidX 25 mL fluid trough* into *Position A*. Add approximately 5 mL of 5% papain solution, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 5% papain solution. Greater volumes may be used, as the numbers listed above are a minimum. Refer to TD-0004.

4.8. Select the "Papain NT Singl" program on the *Integra ViaFlow Pipette*. Refer to TD-0004 for assistance using the *ViaFlow Pipette*. This program will add 30 µL of 5% papain solution to all sample tubes in *Position B* on the *Assist Plus*.

4.9. Once the program is complete, transfer the sample rack back to the *FluidX IntelliXcap*, re-cap the sample tubes, and then replace the safety lid. Slowly invert the sample rack 10 times. Place rack(s) in storage at 4°C for later use, or proceed to sample preparation.

5.0 Sample Preparation Procedure

5.1. Determine the number of sample racks to be analyzed (1-4) and the priority samples needing testing. Reserve a barcoded 384-well assay plate and use this plate barcode as the Batch ID.

5.2. If samples are already at room temperature, proceed to 5.3. For samples stored at 4°C, remove sample rack(s) from the refrigerator. Invert racks 10 times and allow to sit at least 30 minutes at room temperature (up to 2 hours is acceptable).

5.3. Organize the racks numerically by barcode, and assign them numbers 1-4. Then reorganize samples leaving an empty space in each rack for controls, as follows:
- **5.3.1.** rack 1: A2
- **5.3.2.** rack 2: A11
- **5.3.3.** rack 3: H02
- **5.3.4.** rack 4: H11

5.4. Rerack samples to include priority samples and consolidate racks to 95 samples each. If there is not enough space for all priority samples, move some of the original samples to the priority rack to be processed in the next run.

5.5. Invert 10 times, allow to sit for 30 minutes for Papain digestion, inverting an additional 10 times once approximately half-way through.

5.6. After the 30 minute incubation is complete, place sample rack(s) in a 90-95°C water bath for 10 minutes. Then remove samples from the water bath and allow them to cool to room temperature and air dry. Refer to TD-0006.

5.7. Pay special attention during the following steps:

    5.7.1. Ensure that there is sufficient volume and place the COVP1 vial in position A2.

    5.7.2. Replace sample in position A11 in rack 2, as this will be for an NTC.

    5.7.3. Replace sample in position H02 in rack 3, as this will be for an NTC.

    5.7.4. Replace sample in position H11 in rack 4, as this will be for an NTC.

5.8. The reracked tubes now have the samples and control tubes in their final positions. Scan these racks with the *FluidX Perception HD plate scanner* to record the new sample positions.

5.9. Register the reracked sample tubes into the LIMS software.

5.10. Place the clean, barcoded 384-well assay plate, selected in step 5.1, into *Position C* on the *Assist Plus*, and place a clean 25 mL trough in *Position A*.

5.11. Load a rack of 300 µL pipette tips into *Position 1.* Add approximately 5 mL of 1x TBE, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 1x TBE. Greater volumes may be used, as the numbers listed above are a minimum.

5.12. After determining the number of racks to be run (1-4), transfer 25 µL of 1x TBE to the 384-well plate. Refer to TD-0004.

5.13. Have a second person verify the rack barcode and the position of each rack. Then decap tubes and transfer 25 µL of sample from each of the rack(s) to the 384-well plate.

5.14. Manually add 25 µL of positive viral control COVP1 into position A3 of the 384-well plate.

5.15. Seal the 384-well plate(s) with an adhesive plate seal, place into a centrifuge, and spin briefly. Store at 4°C until the qPCR run is ready to begin.

6.0 qPCR Procedure

6.1. Check IntelliQube maintenance log, and perform daily / weekly / monthly maintenance, as needed. See TD-0005 for more information.

6.2. Access the LIMS on workstation 1, generate a .csv sample file as outlined in TD-0007 and name it "XXXXX.csv", where "XXXXX" is the barcode of the 384-well plate.

6.3. Place an *Assay Plate* barcode label A-XXXXX on a LGC 96-well 0.8 mL storage plate. Create the run program in the *IntelliQube software* [TD-0005] and print the final plate layout and required assay mix volumes.

6.4. Load the labeled *Assay Plate* with the assay mixes as per the plate layout.

6.5. Load the 384-well sample plates into the *IntelliQube sample rack hotel* and place the *assay plate* in *Position 1* on the *IntelliQube*. Use the *IntelliQube Interface* to select the appropriate protocol and start the run [TD-0005].

7.0 Results and Reporting

Results from the IntelliQube are automatically sent to Ugentec's FastFinder software and calls are generated. The clinical lab scientist (CLS) will review the results in FastFinder and approve the run. These results will then be available to the Point-n-Click software for release to the medical community. The testing lab will be able to access the results through FastFinder. After data authorization, a list of positive samples and those needing re-testing will be generated. Positive samples will be re-racked and stored in the freezer indefinitely.

8.0 Limitations

8.1. Inadequate amount of sample provided (<500 µL).
8.2. Improper collection of sample.
8.3. Viral load is below assay limit of detection (15 copies / µL).
8.4. Presence of unidentified inhibitors.

9.0 Associated Documents

TD-0001 - Benchling Technical Document
TD-0002 - FluidX Perception™ HD Plate Scanner Technical Document
TD-0003 - Operation of FluidX IntelliXcap 96-Tube Decapper
TD-0004 - Operation of Integra ViaFlo and Assist Plus
TD-0005 - IntelliQube Technical Document
TD-0006 - Operating a Waterbath

REVISION HISTORY

Version Number	Summary of Changes in Current Version	Effective Date
SM-0001.01	New Document	10/27/20

1.0 <u>Objective</u>

The UC Davis High Throughput (UCD HTP) COVID-19 saliva test is intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in saliva samples. This method describes the procedure to prepare, analyze, and evaluate saliva samples for SARS-CoV-2 with multiplexed real-time reverse transcription PCR (rRT-PCR) on a high throughput instrument. For this "Superplex" protocol two fluorescent dyes are used for three probe-based assays in a single reaction. Two SARS-CoV-2 specific assays (the CDC designed nCOV-N1 and nCOV-N2 assays) use probes labeled with the same fluorescent dye (FAM). The human RNA control assay (the CDC designed RNase P assay) employs probes labeled with a second dye (either HEX, QUA670, or Atto-647).

2.0 <u>Reagents and Materials</u>

The following equipment and reagents are required. Equivalent equipment or reagents may be substituted.

2.1. LGC IntelliQube High-throughput qPCR system

2.2. FluidX IntelliXcap 96-tube Decapper, or equivalent

2.3. FluidX Perception™ HD Plate Scanner with IntelliCode software, or equivalent

2.4. Integra ViaFlow 12-channel automated pipette with Assist Plus robotic system, or equivalent

2.5. Integra ViaFlow96 96-channel automated pipette robotic system or Analytik-Jena Cybio Felix liquid handler robot, or equivalent

2.6. Integra 25 mL trough insert; PN #4311, #4312, or equivalent

2.7. Handheld 2D barcode scanner

2.8. 96-well 0.8 mL storage plate; LGC #KBS-7001-131, or equivalent

2.9. 384-well V-bottom plate; Greiner Bio-One #781280, or equivalent

2.10. qPCR master mix:

> LGC RapiDxFire qPCR 5X Master Mix GF 10 mL; LGC #30050-2, and EpiScript RNase H Reverse Transcriptase 25,000 units; LGC #ERT12925K & ERT12925K-1.25ML *or* UltraPlex 1-Step ToughMix (4X); Quantabio #95166, or equivalent

2.11. LGC Biosearch primers and probes [2019-nCoV CDC-qualified Probe and Primer Kits for SARS-CoV-2] or their equivalents [e.g. IDT SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay]:

2.11.1. 2019-nCov CDC Probe and Primer Kit for SAR-CoV-2; LGC #KIT-nCoV-PP1-1000

2.11.2. 2019-nCoV_N2 Reverse Primer - 1000 nmol (NCOV-N2-R-1000)

2.11.3. 2019-nCoV_N2 Forward Primer - 1000 nmol (NCOV-N2-F-1000)

2.11.4. 2019-nCoV_N2 Probe (FAM labeled) - 250 nmol (NCOV-N2-P-250)

2.11.5. Human RNase P Extraction Control Reverse Primer - 1000 nmol (RNP-R-1000)

2.11.6. Human RNase P Extraction Control Forward Primer - 1000 nmol (RNP-F-1000)

2.11.7. Human RNase P Extraction Control Probe (QUA670 labeled) - 250 nmol (RNP-P-250)

2.11.8. 2019-nCoV_N1 Reverse Primer - 1000 nmol (NCOV-N1-R-1000)

2.11.9. 2019-nCoV_N1 Forward Primer - 1000 nmol (NCOV-N1-F-1000)

2.11.10. 2019-nCoV_N1 Probe (FAM labeled) - 250 nmol (NCOV-N1-P-250)

2.11.11. RNAse P oligos (Eurofins or equivalent): RNase P forward and reverse primers in 1X Low-EDTA TE Buffer (100 µM each) and RNase P probe - HEX-labeled in 1X Low-EDTA TE Buffer (100 µM), or equivalent (sequence identical to the LGC and IDT primers and probes above)

2.12. BEI gamma irradiated SARS-CoV-2 virus positive controls; BEI Resources NR-52287

2.13. SuperROX® Reference Dye; Biosearch Technologies #SR-1000, or equivalent

2.14. 10X TBE buffer (Tris, Boric Acid, EDTA); Bio-RAD #1610733, or equivalent

2.15. TE buffer (10 mM Tris, 1 M EDTA, pH 8.0); Invitrogen #AM9849, or equivalent

2.16. Low-EDTA TE buffer (10 mM Tris, 0.1 M EDTA, pH 8.0); Teknova #T0223, or equivalent

2.17. Papain (powder, purified); Spectrum Chemical #PA125, or equivalent

2.18. RiboGuard RNase Inhibitor (40x); Biosearch Technologies #RG90910K, or equivalent

2.19. Water, RNase free, Molecular Biology Grade; Corning #46-000-CM, or equivalent

2.20. Computer, Windows 10, with Internet access and USB3 port

3.0 Preparation of Reagents, Standards, and Sample Solutions

Alternate volumes may be used, as long as the concentration remains the same.

3.1.   1X TBE Solution

Add 100 mL 10X TBE to a clean empty liter bottle, then fill to the 1 L mark with RNase-free water.

3.2.   5% Papain Solution

Weigh out 22.5 g Papain and transfer to a clean empty liter bottle. Fill to 350 mL with 10x TBE Solution. Allow Papain to dissolve, then fill to 450 mL. This solution is stable for 7 days when stored at 4°C.

3.3.   80X Superplex Primer and Probes

3.3.1. Using the 2019-nCoV Primer and Probe kit:

3.3.1.1. The lyophilized 2019-nCoV Primer and Probe come in pre-aliquoted microfuge tubes. <u>Discard the RNase P primer and probe mix tube.</u> Allow the N1 and N2 tubes to reach ambient temperature, then briefly centrifuge using a microcentrifuge. Resuspend the N1 and N2 Primer and Probe mix by adding 90 µL 1X Low-EDTA TE Buffer and vortexing briefly to dissolve. Centrifuge to collect all liquid at the bottom of the tube. Wait at least 20 minutes and centrifuge again.

3.3.1.2. Label a tube "SP Assay 80X" and add the following:
90 µL LGC N1 primer/probe mix
90 µL LGC N2 primer/probe mix
25.2 µL RNase P-F primer (100 µM)
25.2 µL RNase P-R primer (100 µM)
25.2 µL RNase P HEX probe (100 µM)

3.4. Using individual primers and probes

3.4.1. Make stock solutions: Allow lyophilized tubes to come to ambient temperature, then briefly centrifuge using a microcentrifuge. Resuspend all primers in 2.5 ml 1X Low-EDTA TE to 400 µM and probes in 1 ml 1X Low-EDTA TE Buffer to 250 µM. Vortex briefly to dissolve, centrifuge to collect all liquid at the bottom of the tubes, wait at least 20 minutes, vortex and centrifuge again.

3.4.2. After resuspended, dilute the RNase P primers 4X and all probes 2.5X so each has a final concentration of 100 µM.

3.4.3. Label a tube "SP Assay 80X Stock" and add 25 µL of each:
N1 forward primer (400 µM)
N1 reverse primer (400 µM)
N1 probe (100 µM)
N2 forward primer (400 µM)
N2 reverse primer (400 µM)
N2 probe (100 µM)
RNase P forward primer (100 µM)
RNase P reverse primer (100 µM)
RNase P probe (100 µM)
1X Low-EDTA TE Buffer

3.4.4. Vortex the oligos, spin down and store at 4°C, protected from light, for up to a month.

3.5. Assay Mix

** Use the same day or freeze in aliquots, shielded from light. Freeze/thaw less than five times.

Make a mix of PCR reagents using RNase-free water, RapiDxFire qPCR 5X Master Mix GF, EpiScript RNase H Reverse Transcriptase, SuperROX, and Superplex 80X probe mix in a ratio of 0.515 : 0.400 : 0.050 : 0.010 : 0.025. Mix to combine.

  or

Make a mix of PCR reagents using RNase-free water, QuantaBio UltraPlex Master Mix (4X), SuperRox (15 µM), and Superplex 80X probe mix in a 0.465: 0.500 : 0.010 : 0.025 ratio. Mix to combine.

4.0 Control Samples

Positive control (COVP1)   BEI gamma irradiated virus controls diluted in TE buffer/1x Riboguard (or equivalent) to 10,000 copies per microliter. Then added to negative human saliva samples to a final concentration of at least 250 copies per µL.

Negative control (NTC)   1X TBE buffer.

5.0 Receiving Procedure

5.1. Samples will arrive in 1 mL screw-top tubes in a 96-tube SBS format rack.

5.2. Place sample rack(s) in a 80-85°C water bath for 15 min. Place no more than four racks in a water bath at a time. If more than four racks are received, keep the remaining racks in the plastic bin labeled "Raw, non-deactivated saliva" until they are able to be processed. Refer to TD-0006.

5.3. After 15 min. remove sample rack(s) from the water bath and place in a pre-processing bin. Spin upside down in the manual centrifuge to remove fluid from the sample tube caps. Then spin right side up to remove the remaining water out of the rack, and the saliva away from the inside of the tube caps. Remove the rack lids and allow to cool to room temperature and air dry.

5.4. Scan sample tubes with a *FluidX Perception HD plate scanner* as outlined in TD-0002, and register sample information in the LIMS software as described in TD-0007.

5.5. Remove the rack lid and remove the sample tube caps using a *FluidX IntelliXcap* decapper, or equivalent.

5.6. Add 30 µL Papain to each tube of a rack using one of the following methods.

5.6.1. *Integra Assist Plus*

    5.6.1.1. Place the sample rack in *Position B.*

    5.6.1.2. Load a rack of 300 µL pipette tips onto the *Assist Plus Position 1* and a *FluidX 25 mL fluid trough* into *Position A*. Add approximately 5 mL of 5% papain solution, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 5% papain solution. Greater volumes may be used, as the numbers listed above are a minimum. Refer to TD-0004.

    5.6.1.3. Select the "Papain NT Singl" program on the *Integra ViaFlow Pipette*. Refer to TD-0004 for assistance using the *ViaFlow Pipette*. This program will add 30 µL of 5% papain solution to all sample tubes in *Position B* on the *Assist Plus*.

5.6.2. Once the program is complete, transfer the sample rack back to the *FluidX IntelliXcap*, re-cap the sample tubes, and replace the rack lid.

Alternatively, carry out the Papain to saliva samples addition using the CyBio Felix liquid handling robot; follow the on-screen instructions and prompts. (Refer to TD-010.)

5.7. Recap sample tubes using the *FluidX IntelliXcap* decapper or equivalent, and replace the rack lid.

5.8. Slowly invert the sample rack at least 10 times, ensuring the air bubble travels all the way up and down the tube with each inversion.

5.9. Place rack(s) in storage at 4°C for later use, or proceed to step 5.11.

5.10. For samples stored at 4°C, remove sample rack(s) from the refrigerator. Invert racks 10 times, space racks apart from each other and allow to sit at least 30 minutes, but no longer than 2 hours, at room temperature.

5.11. Allow to sit for 30 minutes at room temperature for Papain digestion, inverting an additional 10 times once approximately half-way through.

5.12. After the 30 minute incubation is complete, place sample rack(s) in a 95 $\pm$ 3°C water bath for 10 - 30 minutes. Refer to TD-0006. Then remove samples from the water bath and allow them to cool to room temperature and air dry.

6.0 <u>Sample Preparation Procedure</u>

Determine the number of sample racks to be analyzed (1-4) and the priority samples needing testing. Reserve a barcoded 384-well plate and use this plate barcode as the Batch ID.

6.1. Organize the racks numerically by barcode, and assign them numbers 1-4. Then reorganize samples leaving an empty space in each rack for controls, as follows:

    6.1.1. rack 1: A2

    6.1.2. rack 2: A11

    6.1.3. rack 3: H2

    6.1.4. rack 4: H11

6.2. Rerack samples to include priority samples and consolidate racks to 95 samples each. If there is not enough space for all priority samples, move some of the original samples to the priority rack to be processed in the next run.

6.3. The rearranged racks now have the samples in their final positions, with empty spaces for the controls.

6.4. Scan the sample racks with the *FluidX Perception HD plate scanner* to record the final tube positions and create the 384-well plate in the LIMS. Have a second person verify the 384-well plate was correctly created in the LIMS.

6.5. Load 1X TBE into 384-well plate (Integra Assist Plus)

    6.5.1. Place the clean, barcoded 384-well plate, selected in step 6.0, into *Position C* on the *Assist Plus*, and place a clean 25 mL trough in *Position A*.

    6.5.2. Load a rack of 300 µL pipette tips into Position 1.

    6.5.3. Add at least 17 mL of 1X TBE, to the trough.

    6.5.4. Transfer 25 µL of 1X TBE to the 384-well plate. Refer to TD-0004. Alternatively, carry out the 1X TBE pre-loading with the program "384W TBE Buffer Prefill" on the CyBio Felix; follow the on-screen instructions and prompts.

6.6. Transfer the saliva to the 384-well plate

    6.6.1. Decap the tubes, and for rack 1 add the positive control COVP1 tube to position A2 in the rack.

    6.6.2. Have a second person verify the rack barcode, controls, and the position/orientation of each rack on the instrument used to create the 384-well plate.

6.6.3. Transfer 25 µL of sample from each of the rack(s) to the 384-well plate. Alternatively, carry out the transfer of the digested saliva samples with the program "Saliva Sample Plating" on the CyBio Felix; follow the on-screen instructions and prompts.

6.7. Seal the 384-well plate with an adhesive plate seal, place into a centrifuge, and spin briefly. Store at 4°C until the qPCR run is ready to begin.

7.0 qPCR Procedure

7.1. Check IntelliQube maintenance log, and perform daily / weekly / monthly maintenance, as needed. See TD-0005 for more information.

7.2. Access the LIMS on workstation 1, download the .csv sample file generated by the LIMS, which corresponds to each 384-well plate to be run.

7.3. Place an *Assay Plate* barcode label A-XXXXX on a LGC 96-well 0.8 mL storage plate. Create the run program in the *IntelliQube software* [TD-0005] and print the final plate layout and required assay mix volumes.

7.4. Ensure the IntelliQube has enough assay tape, tape seals, DI water, and bleach for the run.

7.5. Load the labeled *Assay Plate* with the assay mix as per the plate layout.

7.6. Load the 384-well sample plates into the *IntelliQube sample rack hotel* and place the *Assay Plate* in *Position 1* on the *IntelliQube*. Use the *IntelliQube Interface* to select the appropriate protocol and start the run [TD-0005].

8.0 Results and Reporting

Results from the IntelliQube are automatically sent to the LIMS software and Ugentec's FastFinder software (refer to TD-0009), where calls are generated according to the following decision tree:

PC	ROX	N1N2	RP	Result	Action
-	ANY	ANY	ANY	Retest	Retest
+	-	ANY	ANY	Retest	Retest
+	+	+	+	Positive	Report
+	+	+	- or Inc.	Inconclusive	Report
+	+	Inc.	ANY	Inconclusive	Report
+	+	-	+	Negative	Report
+	+	-	- or Inc.	Invalid	Report

The testing lab will be able to access the raw data through FastFinder and the LIMS. The clinical lab scientist (CLS) will review the results in FastFinder to and approve the run. After data authorization, the results will be reconciled with the sample manifest, uploaded to the LIMS, and made available for release to the medical provider.

9.0 Retests and Sample Storage/Retention

Invalid results require collection of a new specimen. A list of positive samples and those needing re-testing will be generated. At least once a day, samples needing re-testing will be pulled and re-run. Positive samples will be re-racked and stored at $\leq$ -20°C indefinitely. After these samples are removed, racks will be moved to post-processing storage and stored at 4°C for two weeks.

10.0 Limitations

10.1. Inadequate amount of sample provided (< 500 µL).

10.2. Improper collection of sample.

10.3. Viral load is below assay limit of detection (15 copies/µL raw saliva; 6 copies per reaction).

10.4. Presence of unidentified inhibitors.

11.0 Associated Documents

TD-0002 - FluidX Perception™ HD Plate Scanner Technical Document

TD-0003 - Operation of FluidX IntelliXcap 96-Tube Decapper

TD-0004 - Operation of Integra ViaFlo and Assist Plus

TD-0005 - IntelliQube Technical Document

TD-0006 - Operating a Water bath

TD-0007 – LIMS Technical Document

TD-0008 – Operation of the ViaFlo96

TD-0009 – Fastfinder software by Ugentec

TD-0010 – Operation of the CyBio Felix

REVISION HISTORY

Version Number	Summary of Changes in Current Version	Effective Date
SM-0001.01	New Document	12/03/20
SM-0002.01	Superplex Protocol	1/1/21

Addendum

Based on research and FastFinder SARS-CoV-2 result data analysis performed in-house, the consensus decision in regard to target results is as follows:

- N1N2 of Cq value below 33.00 will be marked as detected.

- N1N2 of Cq value equal to or above 33.00 will be marked as inconclusive.

- RNaseP of Cq value below 33.00 will be marked as detected.

- RNaseP of Cq value equal to or above 33.00 will be marked as not detected.

FastFinder will generate calls according to the decision tree, refer to 8.0. All invalid and inconclusive results will require collection of a new specimen.

Addendum 2

In an on-going effort to provide the best quality of SARS-CoV-2 RT-qPCR results, all positive samples have been routinely retested after reporting as part of quality assessment. Through this process, it was observed that not all calls with N1N2 Cq ≥31 were reproducible. To address this situation, all samples with N1N2 Cq ≥31 will be rerun within 24 hours prior to reporting. This supersedes the previous N1N2 "inconclusive" category (see Addendum from February 2021). The new reporting scheme for all samples with N1N2 Cq ≥31, but <40, is as follows:

Initial Run	Rerun	Final Result
N1N2 31≤Cq<40	N1N2 Cq<40	Detected
N1N2 31≤Cq<40	Not Detected or Cq>40	Inconclusive

Additionally, in rare cases where the fluorescence of a sample increases above baseline but does not have the normal sigmoidal form, the curve shape and Relative Fluorescence Unit reading can be used to interpret final results, which may differ from the above scheme as defined in Section 8.3 of TD-0009.02.

All inconclusive results will require collection of a new sample.

All samples with Cq <31 will be reported immediately after the initial run as described in the Standard Method.

1. Objective

The UC Davis High Throughput (UCD HTP) COVID-19 saliva test is intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in saliva samples. This method describes the procedure to prepare, analyze, and evaluate saliva samples for SARS-CoV-2 with multiplexed real-time reverse transcription PCR (rRT-PCR) on a high throughput instrument. For this "Superplex" protocol two fluorescent dyes are used for three probe-based assays in a single reaction. Two SARS-CoV-2 specific assays (the CDC designed nCOV-N1 and nCOV-N2 assays) use probes labeled with the same fluorescent dye (FAM). The human RNase P control assay (the CDC designed RNase P assay) employs probes labeled with a second dye (either HEX, QUA670, Atto-647, or Cal Fluor Orange [CFO]).

2. Equipment, Reagents and Materials

The following equipment and reagents are required. Equivalent equipment or reagents may be substituted.

2.1. LGC IntelliQube High-throughput qPCR system

2.2. FluidX IntelliXcap 96-tube Decapper, or equivalent

2.3. FluidX Perception™ HD Plate Scanner with IntelliCode software, or equivalent

2.4. Integra ViaFlow 12-channel automated pipette with Assist Plus robotic system, or equivalent

2.5. Integra ViaFlow96 96-channel automated pipette robotic system or Analytik-Jena Cybio Felix liquid handler robot, or equivalent

2.6. Integra 25 mL trough insert; PN #4311, #4312, or equivalent

2.7. Handheld 2D barcode scanner

2.8. 96-well 0.8 mL storage plate; LGC #KBS-7001-131, or equivalent

2.9. 384-well V-bottom plate; Greiner Bio-One #781280, or equivalent

2.10. qPCR master mix:

LGC RapiDxFire qPCR 5X Master Mix GF 10 mL; LGC #30050-2, and EpiScript RNase H Reverse Transcriptase 25,000 units; LGC #ERT12925K & ERT12925K-1.25ML *or* UltraPlex 1-Step ToughMix (4X); Quantabio #95166, or equivalent

2.11. LGC Biosearch primers and probes (2019-nCoV CDC-qualified Probe and Primer Kits for SARS-CoV-2) or their equivalents (e.g. IDT SARS-CoV-2 [2019-nCoV] CDC qPCR Probe Assay):

2.11.1. 2019-nCov CDC Probe and Primer Kit for SAR-CoV-2; LGC #KIT-nCoV-PP1-1000

2.11.2. 2019-nCoV_N2 Reverse Primer - 1000 nmol (NCOV-N2-R-1000)

2.11.3. 2019-nCoV_N2 Forward Primer - 1000 nmol (NCOV-N2-F-1000)

2.11.4. 2019-nCoV_N2 Probe (FAM labeled) - 250 nmol (NCOV-N2-P-250)

2.11.5. Human RNase P Extraction Control Reverse Primer - 1000 nmol (RNP-R-1000)

2.11.6. Human RNase P Extraction Control Forward Primer - 1000 nmol (RNP-F-1000)

2.11.7. Human RNase P Extraction Control Probe (QUA670 labeled) - 250 nmol (RNP-P-250)

2.11.8. 2019-nCoV_N1 Reverse Primer - 1000 nmol (NCOV-N1-R-1000)

2.11.9. 2019-nCoV_N1 Forward Primer - 1000 nmol (NCOV-N1-F-1000)

2.11.10. 2019-nCoV_N1 Probe (FAM labeled) - 250 nmol (NCOV-N1-P-250)

2.11.11. RNAse P oligos (Eurofins or equivalent): RNase P forward and reverse primers in 1X Low-EDTA TE Buffer (100 µM each) and RNase P probe - HEX-labeled in 1X Low-EDTA TE Buffer (100 µM), or equivalent (sequence identical to the LGC and IDT primers and probes above)

2.11.12. Premixed Superplex probe-set: N1N2RNP-RUO-1010 (CFO labeled)

2.12. BEI gamma irradiated SARS-CoV-2 virus positive controls; BEI Resources e.g. NR-52287

2.13. SuperROX® Reference Dye; Biosearch Technologies #SR-1000, or equivalent

2.14. 10X TBE buffer (Tris, Boric Acid, EDTA); Bio-RAD #1610733, or equivalent

2.15. TE buffer (10 mM Tris, 1 M EDTA, pH 8.0); Invitrogen #AM9849, or equivalent

2.16. Low-EDTA TE buffer (10 mM Tris, 0.1 M EDTA, pH 8.0); Teknova #T0223, or equivalent

2.17. Papain (powder, purified); Spectrum Chemical #PA125, or equivalent

2.18. RiboGuard RNase Inhibitor (40x); Biosearch Technologies #RG90910K, or equivalent

2.19. Water, RNase free, Molecular Biology Grade; Corning #46-000-CM, or equivalent

2.20. Computer, Windows 10, with Internet access and USB3 port

3. <u>Preparation/Use of Reagents and Standards</u>

Alternative volumes may be used, as long as the concentrations remain the same.

3.1. 1X TBE Solution

Add 100 mL 10X TBE to a clean empty liter bottle, and then fill to the 1 L mark with RNase-free water.

3.2. 5% Papain Solution

Weigh out 22.5 g Papain and transfer to a clean empty liter bottle. Fill to 350 mL with 10x TBE Solution. Allow Papain to dissolve, and then fill to 450 mL. This solution is stable for 7 days when stored at 4°C.

3.3. 80X Superplex Primer and Probes

3.3.1. Using the 2019-nCoV Primer and Probe kit:

3.3.1.1. The lyophilized 2019-nCoV Primer and Probe come in pre-aliquoted microfuge tubes. <u>Discard the RNase P primer and probe mix tube.</u> Allow the N1 and N2 tubes to reach ambient temperature, and then briefly centrifuge using a microcentrifuge. Resuspend the N1 and N2 Primer and Probe mix by adding 90 μL 1X Low-EDTA TE Buffer and vortexing briefly to dissolve. Centrifuge to collect all liquid at the bottom of the tube. Wait at least 20 minutes and centrifuge again.

3.3.1.2. Label a tube "SP Assay 80X" and add the following:
  90 μL LGC N1 primer/probe mix
  90 μL LGC N2 primer/probe mix
  25.2 μL RNase P-F primer (100 μM)
  25.2 μL RNase P-R primer (100 μM)
  25.2 μL RNase P HEX probe (100 μM)

3.3.2. Using individual primers and probes:

3.3.2.1. Making stock solutions: Allow lyophilized tubes to come to ambient temperature, and then briefly centrifuge using a microcentrifuge. Resuspend all primers in 2.5 ml 1X Low-EDTA TE to 400 μM and probes in 1 ml 1X Low-EDTA TE Buffer to 250 μM. Vortex briefly to dissolve, centrifuge to collect all liquid at the bottom of the tubes, wait at least 20 minutes, vortex and centrifuge again.

3.3.2.2. After resuspended, dilute the RNase P primers 4X and all probes 2.5X so each has a final concentration of 100 μM.

3.3.2.3. Label a tube "SP Assay 80X Stock" and add 25 μL of each:
N1 forward primer (400 μM)
N1 reverse primer (400 μM)
N1 probe (100 μM)
N2 forward primer (400 μM)
N2 reverse primer (400 μM)
N2 probe (100 μM)
RNase P forward primer (100 μM)
RNase P reverse primer (100 μM)
RNase P probe (100 μM)
1X Low-EDTA TE Buffer

3.3.2.4. Vortex the oligos, spin down and store at 4°C, protected from light, for up to a month.

3.4. Assay Mix

*** Use the same day or freeze in aliquots, shielded from light. Freeze/thaw less than five times***

Make a mix of PCR reagents using RNase-free water, RapiDxFire qPCR 5X Master Mix GF, EpiScript RNase H Reverse Transcriptase, SuperROX, and Superplex 80X probe mix in a ratio of 0.515 : 0.400 : 0.050 : 0.010 : 0.025. If not using Superplex 80X probe mix, add primers and probes individually. Mix to combine.

  or

Make a mix of PCR reagents using RNase-free water, QuantaBio UltraPlex Master Mix (4X), SuperRox (15 µM), and Superplex 80X probe mix in a 0.465: 0.500 : 0.010 : 0.025 ratio. Mix to combine.

3.5. Use of Equivalent Reagents

Validation of Superplex Assay requires the use of an alternative RNase P probe with HEX dye to differentiate from the FAM dye used in the 2019-nCoV CDC nCoV-2 Probe and Primer kit. Probes and primers listed in section 2 contain the same sequence as the CDC nCoV-2 Probe and Primer kit and post analytical validation can be used.

4. <u>Test Controls</u>

Positive Control (COVP1):	BEI γ-irradiated virus controls diluted in TE buffer/1x Riboguard (or equivalent) to 10,000 copies per µL, then added to negative human saliva samples to a final concentration of at least 250 copies per µL
Negative Control (No Template Control [NTC]):	1X TBE buffer
Internal Process Control (IPC):	Human RNase P
Passive Reference Control:	Super-ROX dye in every reaction well

5. <u>Sample Receiving Procedure</u>

5.1. Samples arrive in 1 mL screw-top tubes in a 96-tube SBS format rack.

5.2. Place sample rack(s) in a 80-85°C water bath for 15 min. Place no more than four racks in a water bath at a time. If more than four racks are received, keep the remaining racks in the plastic bin labeled "Raw, non-deactivated saliva" until they are able to be processed. Refer to TD-0006.

5.3. After 15 min. remove sample rack(s) from the water bath and place in a pre-processing bin. Spin upside down in the manual centrifuge to remove fluid from the

sample tube caps. Then spin right side up to remove the remaining water out of the rack, and the saliva away from the inside of the tube caps. Remove the rack lids and allow cooling to room temperature for approximately ten minutes and air dry.

5.4. Scan sample tubes with a *FluidX Perception HD plate scanner* as outlined in TD-0002, and register sample information in the LIMS software as described in TD-0007.

5.5. Remove the rack lid and remove the sample tube caps using a *FluidX IntelliXcap* decapper, or equivalent.

5.6. Add 30 µL Papain to each tube of a rack using one of the following methods.
    5.6.1. *Integra Assist Plus*
        5.6.1.1. Place the sample rack in *Position B*.
        5.6.1.2. Load a rack of 300 µL pipette tips onto the *Assist Plus Position 1* and a *FluidX 25 mL fluid trough* into *Position A*. Add approximately 5 mL of 5% Papain solution, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 5% Papain solution. Greater volumes may be used, as the numbers listed above are a minimum. Refer to TD-0004.
        5.6.1.3. Select the "Papain NT Singl" program on the *Integra ViaFlow Pipette*. Refer to TD-0004 for assistance using the *ViaFlow Pipette*. This program will add 30 µL of 5% Papain solution to all sample tubes in *Position B* on the *Assist Plus*.
    5.6.2. Once the program is complete, transfer the sample rack back to the *FluidX IntelliXcap*, re-cap the sample tubes, and replace the rack lid.
    5.6.3. Alternatively, carry out the Papain to saliva samples addition using the CyBio Felix liquid handling robot; follow the on-screen instructions and prompts (refer to TD-0010).

5.7. Place rack(s) in storage at 4°C for later use, or proceed to step 5.9

5.8. For samples stored at 4°C, remove sample rack(s) from the refrigerator. Space racks apart from each other and allow to sit at room temperature for a minimum of 30 minutes, but no longer than 2 hours.

5.9. For Papain digestion, place racks on either Shaker 1 or Shaker 2 (maximum 8 racks per shaker), start a timer for 30 minutes, and turn on Shaker to mix. When five minute mixing program is complete, allow samples to sit for the remainder of the 30 minutes at room temperature.

5.10. After the 30 minute incubation is complete, place sample rack(s) in a 95 $\pm$ 3°C water bath for 20 minutes. Refer to TD-0006. Then remove samples from the water bath and allow them to cool to room temperature and air dry.

6. <u>**Sample Preparation Procedure**</u>

Determine the number of sample racks to be analyzed (1-4) and the priority samples needing testing. Reserve a barcoded 384-well plate and use this plate barcode as the Batch ID.

6.1. Organize the racks numerically by barcode, and assign them numbers 1-4. Then reorganize samples leaving an empty space in each rack for controls, as follows:
- **6.1.1.** rack 1: A2
- **6.1.2.** rack 2: A11
- **6.1.3.** rack 3: H2
- **6.1.4.** rack 4: H11

6.2. Rerack samples to include priority samples and consolidate racks to 95 samples each. If there is not enough space for all priority samples, move some of the original samples to the priority rack to be processed in the next run.

6.3. The rearranged racks now have the samples in their final positions, with empty spaces for the controls.

6.4. Scan the sample racks with the *FluidX Perception HD plate scanner* to record the final tube positions and create the 384-well plate in the LIMS. Have a second person verify the 384-well plate was correctly created in the LIMS.

6.5. Load 1X TBE into 384-well plate (Integra Assist Plus)
- **6.5.1.** Place the clean, barcoded 384-well plate, selected in step 6.0, into *Position C* on the *Assist Plus*, and place a clean 25 mL trough in *Position A*.
- **6.5.2.** Load a rack of 300 µL pipette tips into Position 1.
- **6.5.3.** Add at least 17 mL of 1X TBE, to the trough.
- **6.5.4.** Transfer 25 µL of 1X TBE to the 384-well plate. Refer to TD-0004.
- **6.5.5.** Alternatively, carry out the 1X TBE pre-loading with the program "384W TBE Buffer Prefill" on the CyBio Felix; follow the on-screen instructions and prompts.

6.6. Transfer the saliva to the 384-well plate
- **6.6.1.** Decap the tubes, and for rack 1 add the positive control COVP1 tube to position A2 in the rack.

6.6.2. Have a second person verify the rack barcode, controls, and the position/orientation of each rack on the instrument used to create the 384-well plate.

6.6.3. Transfer 25 µL of sample from each of the rack(s) to the 384-well plate using CyBio Felix and follow the on-screen instructions and prompts (see TD-0010).

6.6.4. Alternatively, the Integra Viaflow 96 may be used as a backup, if needed.

6.7. Seal the 384-well plate with an adhesive plate seal, place into a centrifuge, and spin briefly. Store at RT until the qPCR run is ready to begin. If plate needs to be stored for longer than 4 hours, place in -20°C freezer.

7. qPCR Procedure

7.1. Check IntelliQube maintenance log, and perform daily / weekly / monthly maintenance, as needed. See TD-0005 for more information.

7.2. Access the LIMS on workstation 1 or laptop connected to Genome Center network and download the .csv sample file generated by the LIMS, which corresponds to each 384-well plate to be run.

7.3. Place an *Assay Plate* barcode label A-XXXXX on a LGC 96-well 0.8 mL storage plate. Create the run program in the *IntelliQube software* (refer to TD-0005) and print the final plate layout and required assay mix volumes.

7.4. Ensure the IntelliQube has enough assay tape, tape seals, DI water, and bleach for the run.

7.5. Load the labeled *Assay Plate* with the assay mix as per the plate layout.

7.6. Load the 384-well sample plates into the *IntelliQube sample rack hotel* and place the *Assay Plate* in *Position 1* on the *IntelliQube*. Use the *IntelliQube Interface* to select the appropriate protocol and start the run (refer to TD-0005).

8. Results and Reporting

8.1. Results from the IntelliQube are automatically sent to the LIMS software and Ugentec's FastFinder software (refer to TD-0009), where calls are generated according to the following decision tree:

PC	ROX	N1N2	RP	Result	Action
-	ANY	ANY	ANY	Retest	Retest
+	-	ANY	ANY	Retest	Retest
+	+	+	+	Positive	Report
+	+	+	- or Inc.	Inconclusive	Report
+	+	Inc.	ANY	Inconclusive	Report
+	+	-	+	Negative	Report
+	+	-	- or Inc.	Invalid	Report

8.2. The following applies to RNase P target resulting:

8.2.1. RNase P of Cq value < 33.00 will be marked as **detected.**

8.2.2. RNase P of Cq value $\geq$ 33.00 will be marked as **not detected.**

8.3. All samples with N1N2 Cq ≥31 will be rerun within 24 hours prior to reporting. The reporting scheme for all samples with N1N2 Cq ≥31, but <40, is as follows:

Initial Run	Rerun	Final Result
N1N2 31≤Cq<40	N1N2 Cq<40	**Detected**
N1N2 31≤Cq<40	Not Detected or Cq>40	**Inconclusive**

8.3.1. Additionally, in rare cases where the fluorescence of a sample increases above baseline but does not have the normal sigmoidal form, the curve shape and Relative Fluorescence Unit (RFU) reading can be used to interpret final results, which may differ from the above scheme, as defined in Section 8.3 of TD-0009.02.

8.3.2. All samples with Cq <31 will be reported immediately after the initial run.

8.4. The testing lab will be able to access the raw data through FastFinder and the LIMS. The clinical lab scientist (CLS) will review the results in FastFinder to approve the run. After data authorization, the results will be reconciled with the sample manifest, uploaded to the LIMS, and made available for release to the medical provider.

9. **Retests and Sample Storage/Retention**

   9.1. Invalid and inconclusive results require collection of a new specimen.

   9.2. A list of positive samples and those needing re-testing will be generated. At least once a day, samples needing re-testing will be pulled and samples re-run.

   9.3. Positive samples will be re-racked and stored at $\leq$ -20°C indefinitely.

   9.4. After positive samples are removed, racks will be moved to post-processing storage and stored at 4°C for two weeks.

10. **Limitations**

    10.1. Inadequate amount of sample provided (< 500 µL)

    10.2. Improper collection of sample

    10.3. Viral load is below assay limit of detection (12.5 copies/µL raw saliva; 5 copies per reaction)

    10.4. Presence of unidentified inhibitors.

11. **Associated Documents**

    TD-0002 - FluidX Perception™ HD Plate Scanner Technical Document
    TD-0003 - Operation of FluidX IntelliXcap 96-Tube Decapper
    TD-0004 - Operation of Integra ViaFlo and Assist Plus
    TD-0005 - IntelliQube Technical Document
    TD-0006 - Operating a Water bath
    TD-0007 - LIMS Technical Document
    TD-0008 - Operation of the ViaFlo96
    TD-0009 - FastFinder software by Ugentec
    TD-0010 - Operation of the CyBio Felix

REVISION HISTORY

Version Number	Summary of Changes in Current Version	Effective Date
SM-0002.02	Superplex Protocol	6/11/21

1.0 Objective

The UC Davis High Throughput (UCD HTP) COVID-19 saliva test is intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in saliva samples. This method describes the procedure to prepare, analyze, and evaluate saliva samples for SARS-CoV-2 with real-time reverse transcription PCR (rRT-PCR) on a high throughput instrument.

2.0 Reagents and Materials

The following equipment and reagents are required. Equivalent equipment or reagents may be substituted.

- **2.1.** LGC IntelliQube High-throughput qPCR system
- **2.2.** FluidX IntelliXcap 96-tube Decapper or equivalent
- **2.3.** FluidX Perception™ HD Plate Scanner with IntelliCode software
- **2.4.** Integra 25 mL trough insert; PN#4311, 4312 or equivalent
- **2.5.** Integra ViaFlow 12-channel automated pipettes with Assist Plus robotic system, a Viaflo-96, or equivalent
- **2.6.** Analytical balance
- **2.7.** Handheld 2D barcode scanner
- **2.8.** Micropipettes 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL
- **2.9.** Graduated cylinder and serological pipettes
- **2.10.** 96-well 0.8 mL storage plate; LGC #KBS-7001-131
- **2.11.** 384-well V-bottom plate; Greiner Bio-One #781280
- **2.12.** qPCR master mix:
      LGC RapiDxFire qPCR 5X Master Mix GF 10 mL; LGC #30050-2, and EpiScript RNase H Reverse Transcriptase 25,000 units; LGC #ERT12925K
      or
      UltraPlex 1-Step ToughMix (4X); Quantabio #95166 or equivalent
- **2.13.** 2019-nCov CDC Probe and Primer Kit for SAR-CoV-2; LGC #KIT-nCoV-PP1-1000 or equivalent.
- **2.14.** BEI gamma irradiated, SARS-CoV-2 virus; BEI Resources NR-52287
- **2.15.** SuperROX® Reference Dye; Biosearch Technologies #SR-1000
- **2.16.** 10x TBE buffer (Tris, Boric Acid, EDTA); Bio-RAD #1610733 or equivalent
- **2.17.** TE buffer (10 mM Tris, 1 M EDTA, pH 8.0); Invitrogen #AM9849 or equivalent
- **2.18.** Low-EDTA TE buffer (10 mM Tris, 0.1 M EDTA, pH 8.0); Teknova #T0223 or equivalent.
- **2.19.** Papain (powder, purified); Spectrum Chemical #PA125.
- **2.20.** RiboGuard RNase Inhibitor (40x); Biosearch Technologies #RG90910K or equivalent.
- **2.21.** Water, RNase free, Molecular Biology Grade; Corning #46-000-CM or equivalent.

2.22. Computer, Windows 10, with Internet access and USB3 port.

3.0 <u>Preparation of Reagents, Standards, and Sample Solutions</u>
Alternate volumes may be used, as long as the concentration remains the same.

3.1. 1X TBE Solution
Add 5 mL 10X TBE in a graduated cylinder, then fill to the 50 mL mark with RNase-free water.

3.2. 5% Papain Solution
Weigh out 2.25 g Papain and transfer to a 50 mL conical tube. Fill to 35 mL with 10x TBE Solution. Allow Papain to dissolve, then fill to 45 mL. This solution is stable for 7 days when stored at 4°C.

3.3. 80x 2019-nCoV Primer and Probe
The lyophilized 2019-nCoV Primer and Probe come in pre-aliquoted microfuge tubes. Allow these tubes to reach ambient temperature, then briefly centrifuge for 10 seconds using a microcentrifuge. Resuspend each Primer and Probe mix (N1, N2 and RNase P) by adding 250 µl 1X Low-EDTA TE Buffer and vortexing briefly to dissolve. Centrifuge again to collect all liquid at the bottom of the tube. Store at 4°C, protected from light, for up to a month.

3.4. N2 Spiked Probe Mix
Transfer 51.3 µL of the N2 Probe Mix to a clean microtube and add 7 µL 200 µM N2F Primer and 7 µL 200 µM N2R Primer. Pipette up and down to mix. Store at 4°C, protected from light, for up to a month.

3.5. Assay Mix
Make one large mix of PCR reagents for all three assays using RapiDxFire qPCR 5X Master Mix GF, EpiScript RNase H Reverse Transcriptase, SuperROX, and RNase-free water in a ratio of 0.400 : 0.050 : 0.010 : 0.515. Mix to combine, then divide into thirds. Add the appropriate Primer and Probe mix at the following ratio: N1 (0.025), Spiked N2 (0.030), and RNase P (0.025). Prepare fresh daily and shield from light
    or
Make one large mix of PCR reagents for all three assays using QuantaBio UltraPlex Master Mix (4X), SuperRox (15uM), and water in a 0.500 : 0.010 : 0.465 ratio. For example, 232.2 µL of MasterMix, 4.6 µL SuperRox, and 2.15.9 µL water. Mix to combine then divide into thirds. Prepare fresh daily.

3.6. Control Samples

COVP1 control:	BEI gamma irradiated virus controls diluted in TE buffer/1x Riboguard (or equivalent) to 10,000 copies per microliter. Then added to negative human saliva samples to a final concentration of 250 copies per µL.
NTC (no template control):	1x TBE buffer.

4.0 <u>Receiving Procedure</u>

4.1. Samples will arrive in 1 mL screw-top tubes in a 96-tube format.

4.2. Place sample rack(s) in an 80-85°C water bath for 15 min. Place up to four racks in the water bath at a time. If we receive more than four racks, keep the remaining racks in the plastic tub labeled "Non-deactivated saliva" until they are able to be processed. Refer to TD-0006.

4.3. After 15 min. remove sample rack(s) from the water bath. Place paper towels or other absorbent material between the sample tube caps and sample rack lid, and shake to remove excess fluid from the sample tube caps. Then remove the rack lid and allow to cool to room temperature and air dry.

4.4. Sample tubes are then scanned with a *FluidX Perception HD plate scanner*, and sample information linked to a specific rack and position as outlined in TD-0002.

4.5. After the sample tubes have been linked to their respective rack in the LIMS software in TD-0001, transfer the entire rack to the *FluidX IntelliXcap*, remove the rack safety lid, and remove the caps from the sample tubes as outlined in TD-0003.

4.6. After decapping, move the sample rack to the *Integra Assist Plus* in *Position B*.

4.7. Load a rack of 300 µL pipette tips onto the *Assist Plus Position 1* and the *FluidX 25 mL fluid trough* into *Position A*. Add approximately 5 mL of 5% papain solution, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 5% papain solution. Greater volumes may be used, as the numbers listed above are a minimum. Refer to TD-0004.

4.8. Select the "Papain NT Singl" program on the *Integra ViaFlow Pipette*. Refer to TD-0004 for assistance using the *ViaFlow Pipette*. This program will add 30 µL of 5% papain solution to all sample tubes in *Position B* on the *Assist Plus.*

4.9. Once the program is complete, transfer the sample rack back to the *FluidX IntelliXcap*, re-cap the sample tubes, and then replace the safety lid. Slowly invert the sample rack 10 times. Place rack(s) in storage at 4°C for later use, or proceed to sample preparation.

5.0 Sample Preparation Procedure

5.1. Determine the number of sample racks to be analyzed (1-4) and the priority samples needing testing. Reserve a barcoded 384-well assay plate and use this plate barcode as the Batch ID.

5.2. If samples are already at room temperature, proceed to 5.3. For samples stored at 4°C, remove sample rack(s) from the refrigerator. Invert racks 10 times and allow to sit at least 30 minutes at room temperature (up to 2 hours is acceptable).

5.3. Organize the racks numerically by barcode, and assign them numbers 1-4. Then reorganize samples leaving an empty space in each rack for controls, as follows:
  5.3.1. rack 1: A2
  5.3.2. rack 2: A11
  5.3.3. rack 3: H02
  5.3.4. rack 4: H11

5.4. Rerack samples to include priority samples and consolidate racks to 95 samples each. If there is not enough space for all priority samples, move some of the original samples to the priority rack to be processed in the next run.

5.5. Invert 10 times, allow to sit for 30 minutes for Papain digestion, inverting an additional 10 times once approximately half-way through.

5.6. After the 30 minute incubation is complete, place sample rack(s) in a 90-95°C water bath for 10 minutes. Then remove samples from the water bath and allow them to cool to room temperature and air dry. Refer to TD-0006.

5.7. Pay special attention during the following steps:

5.7.1. Ensure that there is sufficient volume and place the COVP1 vial in position A2.

5.7.2. Replace sample in position A11 in rack 2, as this will be for an NTC.

5.7.3. Replace sample in position H02 in rack 3, as this will be for an NTC.

5.7.4. Replace sample in position H11 in rack 4, as this will be for an NTC.

5.8. The reracked tubes now have the samples and control tubes in their final positions. Scan these racks with the *FluidX Perception HD plate scanner* to record the new sample positions.

5.9. Register the reracked sample tubes into the LIMS software.

5.10. Place the clean, barcoded 384-well assay plate, selected in step 5.1, into *Position C* on the *Assist Plus*, and place a clean 25 mL trough in *Position A*.

5.11. Load a rack of 300 µL pipette tips into *Position 1.* Add approximately 5 mL of 1x TBE, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 1x TBE. Greater volumes may be used, as the numbers listed above are a minimum.

5.12. After determining the number of racks to be run (1-4), transfer 25 µL of 1x TBE to the 384-well plate. Refer to TD-0004.

5.13. Have a second person verify the rack barcode and the position of each rack. Then decap tubes and transfer 25 µL of sample from each of the rack(s) to the 384-well plate.

5.14. Manually add 25 µL of positive viral control COVP1 into position A3 of the 384-well plate.

5.15. Seal the 384-well plate(s) with an adhesive plate seal, place into a centrifuge, and spin briefly. Store at 4°C until the qPCR run is ready to begin.

6.0 qPCR Procedure

6.1. Check IntelliQube maintenance log, and perform daily / weekly / monthly maintenance, as needed. See TD-0005 for more information.

6.2. Access the LIMS on workstation 1, generate a .csv sample file as outlined in TD-0007 and name it "XXXXX.csv", where "XXXXX" is the barcode of the 384-well plate.

6.3. Place an *Assay Plate* barcode label A-XXXXX on a LGC 96-well 0.8 mL storage plate. Create the run program in the *IntelliQube software* [TD-0005] and print the final plate layout and required assay mix volumes.

6.4. Load the labeled *Assay Plate* with the assay mixes as per the plate layout.

6.5. Load the 384-well sample plates into the *IntelliQube sample rack hotel* and place the *assay plate* in *Position 1* on the *IntelliQube*. Use the *IntelliQube Interface* to select the appropriate protocol and start the run [TD-0005].

7.0 Results and Reporting

Results from the IntelliQube are automatically sent to Ugentec's FastFinder software and calls are generated. The clinical lab scientist (CLS) will review the results in FastFinder and approve the run. These results will then be available to the Point-n-Click software for release to the medical community. The testing lab will be able to access the results through FastFinder. After data authorization, a list of positive samples and those needing re-testing will be generated. Positive samples will be re-racked and stored in the freezer indefinitely.

8.0 Limitations

8.1. Inadequate amount of sample provided (<500 µL).
8.2. Improper collection of sample.
8.3. Viral load is below assay limit of detection (15 copies / µL).
8.4. Presence of unidentified inhibitors.

9.0 Associated Documents

TD-0001 - Benchling Technical Document
TD-0002 - FluidX Perception™ HD Plate Scanner Technical Document
TD-0003 - Operation of FluidX IntelliXcap 96-Tube Decapper
TD-0004 - Operation of Integra ViaFlo and Assist Plus
TD-0005 - IntelliQube Technical Document
TD-0006 - Operating a Waterbath

REVISION HISTORY

Version Number	Summary of Changes in Current Version	Effective Date
SM-0001.01	New Document	10/27/20

1.0 <u>Objective</u>

The UC Davis High Throughput (UCD HTP) COVID-19 saliva test is intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in saliva samples. This method describes the procedure to prepare, analyze, and evaluate saliva samples for SARS-CoV-2 with multiplexed real-time reverse transcription PCR (rRT-PCR) on a high throughput instrument. For this "Superplex" protocol two fluorescent dyes are used for three probe-based assays in a single reaction. Two SARS-CoV-2 specific assays (the CDC designed nCOV-N1 and nCOV-N2 assays) use probes labeled with the same fluorescent dye (FAM). The human RNA control assay (the CDC designed RNase P assay) employs probes labeled with a second dye (either HEX, QUA670, or Atto-647).

2.0 <u>Reagents and Materials</u>

The following equipment and reagents are required. Equivalent equipment or reagents may be substituted.

2.1. LGC IntelliQube High-throughput qPCR system

2.2. FluidX IntelliXcap 96-tube Decapper, or equivalent

2.3. FluidX Perception™ HD Plate Scanner with IntelliCode software, or equivalent

2.4. Integra ViaFlow 12-channel automated pipette with Assist Plus robotic system, or equivalent

2.5. Integra ViaFlow96 96-channel automated pipette robotic system or Analytik-Jena Cybio Felix liquid handler robot, or equivalent

2.6. Integra 25 mL trough insert; PN #4311, #4312, or equivalent

2.7. Handheld 2D barcode scanner

2.8. 96-well 0.8 mL storage plate; LGC #KBS-7001-131, or equivalent

2.9. 384-well V-bottom plate; Greiner Bio-One #781280, or equivalent

2.10. qPCR master mix:

LGC RapiDxFire qPCR 5X Master Mix GF 10 mL; LGC #30050-2, and EpiScript RNase H Reverse Transcriptase 25,000 units; LGC #ERT12925K & ERT12925K-1.25ML *or* UltraPlex 1-Step ToughMix (4X); Quantabio #95166, or equivalent

2.11. LGC Biosearch primers and probes [2019-nCoV CDC-qualified Probe and Primer Kits for SARS-CoV-2] or their equivalents [e.g. IDT SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay]:

    2.11.1. 2019-nCov CDC Probe and Primer Kit for SAR-CoV-2; LGC #KIT-nCoV-PP1-1000

    2.11.2. 2019-nCoV_N2 Reverse Primer - 1000 nmol (NCOV-N2-R-1000)

    2.11.3. 2019-nCoV_N2 Forward Primer - 1000 nmol (NCOV-N2-F-1000)

    2.11.4. 2019-nCoV_N2 Probe (FAM labeled) - 250 nmol (NCOV-N2-P-250)

2.11.5. Human RNase P Extraction Control Reverse Primer - 1000 nmol (RNP-R-1000)

2.11.6. Human RNase P Extraction Control Forward Primer - 1000 nmol (RNP-F-1000)

2.11.7. Human RNase P Extraction Control Probe (QUA670 labeled) - 250 nmol (RNP-P-250)

2.11.8. 2019-nCoV_N1 Reverse Primer - 1000 nmol (NCOV-N1-R-1000)

2.11.9. 2019-nCoV_N1 Forward Primer - 1000 nmol (NCOV-N1-F-1000)

2.11.10. 2019-nCoV_N1 Probe (FAM labeled) - 250 nmol (NCOV-N1-P-250)

2.11.11. RNAse P oligos (Eurofins or equivalent): RNase P forward and reverse primers in 1X Low-EDTA TE Buffer (100 µM each) and RNase P probe - HEX-labeled in 1X Low-EDTA TE Buffer (100 µM), or equivalent (sequence identical to the LGC and IDT primers and probes above)

2.12. BEI gamma irradiated SARS-CoV-2 virus positive controls; BEI Resources NR-52287

2.13. SuperROX® Reference Dye; Biosearch Technologies #SR-1000, or equivalent

2.14. 10X TBE buffer (Tris, Boric Acid, EDTA); Bio-RAD #1610733, or equivalent

2.15. TE buffer (10 mM Tris, 1 M EDTA, pH 8.0); Invitrogen #AM9849, or equivalent

2.16. Low-EDTA TE buffer (10 mM Tris, 0.1 M EDTA, pH 8.0); Teknova #T0223, or equivalent

2.17. Papain (powder, purified); Spectrum Chemical #PA125, or equivalent

2.18. RiboGuard RNase Inhibitor (40x); Biosearch Technologies #RG90910K, or equivalent

2.19. Water, RNase free, Molecular Biology Grade; Corning #46-000-CM, or equivalent

2.20. Computer, Windows 10, with Internet access and USB3 port

3.0 Preparation of Reagents, Standards, and Sample Solutions

Alternate volumes may be used, as long as the concentration remains the same.

3.1. 1X TBE Solution

Add 100 mL 10X TBE to a clean empty liter bottle, then fill to the 1 L mark with RNase-free water.

3.2. 5% Papain Solution

Weigh out 22.5 g Papain and transfer to a clean empty liter bottle. Fill to 350 mL with 10x TBE Solution. Allow Papain to dissolve, then fill to 450 mL. This solution is stable for 7 days when stored at 4°C.

3.3. 80X Superplex Primer and Probes

3.3.1. Using the 2019-nCoV Primer and Probe kit:

3.3.1.1. The lyophilized 2019-nCoV Primer and Probe come in pre-aliquoted microfuge tubes. <u>Discard the RNase P primer and probe mix tube.</u> Allow the N1 and N2 tubes to reach ambient temperature, then briefly centrifuge using a microcentrifuge. Resuspend the N1 and N2 Primer and Probe mix by adding 90 µL 1X Low-EDTA TE Buffer and vortexing briefly to dissolve. Centrifuge to collect all liquid at the bottom of the tube. Wait at least 20 minutes and centrifuge again.

3.3.1.2. Label a tube "SP Assay 80X" and add the following:
90 µL LGC N1 primer/probe mix
90 µL LGC N2 primer/probe mix
25.2 µL RNase P-F primer (100 µM)
25.2 µL RNase P-R primer (100 µM)
25.2 µL RNase P HEX probe (100 µM)

3.4. Using individual primers and probes

3.4.1. Make stock solutions: Allow lyophilized tubes to come to ambient temperature, then briefly centrifuge using a microcentrifuge. Resuspend all primers in 2.5 ml 1X Low-EDTA TE to 400 µM and probes in 1 ml 1X Low-EDTA TE Buffer to 250 µM. Vortex briefly to dissolve, centrifuge to collect all liquid at the bottom of the tubes, wait at least 20 minutes, vortex and centrifuge again.

3.4.2. After resuspended, dilute the RNase P primers 4X and all probes 2.5X so each has a final concentration of 100 µM.

3.4.3. Label a tube "SP Assay 80X Stock" and add 25 µL of each:
N1 forward primer (400 µM)
N1 reverse primer (400 µM)
N1 probe (100 µM)
N2 forward primer (400 µM)
N2 reverse primer (400 µM)
N2 probe (100 µM)
RNase P forward primer (100 µM)
RNase P reverse primer (100 µM)
RNase P probe (100 µM)
1X Low-EDTA TE Buffer

3.4.4. Vortex the oligos, spin down and store at 4°C, protected from light, for up to a month.

3.5. Assay Mix

** Use the same day or freeze in aliquots, shielded from light. Freeze/thaw less than five times.

Make a mix of PCR reagents using RNase-free water, RapiDxFire qPCR 5X Master Mix GF, EpiScript RNase H Reverse Transcriptase, SuperROX, and Superplex 80X probe mix in a ratio of 0.515 : 0.400 : 0.050 : 0.010 : 0.025. Mix to combine.

   or

Make a mix of PCR reagents using RNase-free water, QuantaBio UltraPlex Master Mix (4X), SuperRox (15 µM), and Superplex 80X probe mix in a 0.465: 0.500 : 0.010 : 0.025 ratio. Mix to combine.

4.0 Control Samples

Positive control (COVP1)   BEI gamma irradiated virus controls diluted in TE buffer/1x Riboguard (or equivalent) to 10,000 copies per microliter. Then added to negative human saliva samples to a final concentration of at least 250 copies per µL.

Negative control (NTC)   1X TBE buffer.

5.0 Receiving Procedure

5.1. Samples will arrive in 1 mL screw-top tubes in a 96-tube SBS format rack.

5.2. Place sample rack(s) in a 80-85°C water bath for 15 min. Place no more than four racks in a water bath at a time. If more than four racks are received, keep the remaining racks in the plastic bin labeled "Raw, non-deactivated saliva" until they are able to be processed. Refer to TD-0006.

5.3. After 15 min. remove sample rack(s) from the water bath and place in a pre-processing bin. Spin upside down in the manual centrifuge to remove fluid from the sample tube caps. Then spin right side up to remove the remaining water out of the rack, and the saliva away from the inside of the tube caps. Remove the rack lids and allow to cool to room temperature and air dry.

5.4. Scan sample tubes with a *FluidX Perception HD plate scanner* as outlined in TD-0002, and register sample information in the LIMS software as described in TD-0007.

5.5. Remove the rack lid and remove the sample tube caps using a *FluidX IntelliXcap* decapper, or equivalent.

5.6. Add 30 µL Papain to each tube of a rack using one of the following methods.

5.6.1. *Integra Assist Plus*

5.6.1.1. Place the sample rack in *Position B*.

5.6.1.2. Load a rack of 300 µL pipette tips onto the *Assist Plus Position 1* and a *FluidX 25 mL fluid trough* into *Position A*. Add approximately 5 mL of 5% papain solution, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 5% papain solution. Greater volumes may be used, as the numbers listed above are a minimum. Refer to TD-0004.

5.6.1.3. Select the "Papain NT Singl" program on the *Integra ViaFlow Pipette*. Refer to TD-0004 for assistance using the *ViaFlow Pipette*. This program will add 30 µL of 5% papain solution to all sample tubes in *Position B* on the *Assist Plus*.

5.6.2. Once the program is complete, transfer the sample rack back to the *FluidX IntelliXcap*, re-cap the sample tubes, and replace the rack lid.

Alternatively, carry out the Papain to saliva samples addition using the CyBio Felix liquid handling robot; follow the on-screen instructions and prompts. (Refer to TD-010.)

5.7. Recap sample tubes using the *FluidX IntelliXcap* decapper or equivalent, and replace the rack lid.

5.8. Slowly invert the sample rack at least 10 times, ensuring the air bubble travels all the way up and down the tube with each inversion.

5.9. Place rack(s) in storage at 4°C for later use, or proceed to step 5.11.

5.10. For samples stored at 4°C, remove sample rack(s) from the refrigerator. Invert racks 10 times, space racks apart from each other and allow to sit at least 30 minutes, but no longer than 2 hours, at room temperature.

5.11. Allow to sit for 30 minutes at room temperature for Papain digestion, inverting an additional 10 times once approximately half-way through.

5.12. After the 30 minute incubation is complete, place sample rack(s) in a 95 $\pm$ 3°C water bath for 10 - 30 minutes. Refer to TD-0006. Then remove samples from the water bath and allow them to cool to room temperature and air dry.

6.0 Sample Preparation Procedure

Determine the number of sample racks to be analyzed (1-4) and the priority samples needing testing. Reserve a barcoded 384-well plate and use this plate barcode as the Batch ID.

6.1. Organize the racks numerically by barcode, and assign them numbers 1-4. Then reorganize samples leaving an empty space in each rack for controls, as follows:
  - **6.1.1.** rack 1: A2
  - **6.1.2.** rack 2: A11
  - **6.1.3.** rack 3: H2
  - **6.1.4.** rack 4: H11

6.2. Rerack samples to include priority samples and consolidate racks to 95 samples each. If there is not enough space for all priority samples, move some of the original samples to the priority rack to be processed in the next run.

6.3. The rearranged racks now have the samples in their final positions, with empty spaces for the controls.

6.4. Scan the sample racks with the *FluidX Perception HD plate scanner* to record the final tube positions and create the 384-well plate in the LIMS. Have a second person verify the 384-well plate was correctly created in the LIMS.

6.5. Load 1X TBE into 384-well plate (Integra Assist Plus)
  - **6.5.1.** Place the clean, barcoded 384-well plate, selected in step 6.0, into *Position C* on the *Assist Plus*, and place a clean 25 mL trough in *Position A*.
  - **6.5.2.** Load a rack of 300 µL pipette tips into Position 1.
  - **6.5.3.** Add at least 17 mL of 1X TBE, to the trough.
  - **6.5.4.** Transfer 25 µL of 1X TBE to the 384-well plate. Refer to TD-0004. Alternatively, carry out the 1X TBE pre-loading with the program "384W TBE Buffer Prefill" on the CyBio Felix; follow the on-screen instructions and prompts.

6.6. Transfer the saliva to the 384-well plate
  - **6.6.1.** Decap the tubes, and for rack 1 add the positive control COVP1 tube to position A2 in the rack.
  - **6.6.2.** Have a second person verify the rack barcode, controls, and the position/orientation of each rack on the instrument used to create the 384-well plate.

6.6.3. Transfer 25 μL of sample from each of the rack(s) to the 384-well plate. Alternatively, carry out the transfer of the digested saliva samples with the program "Saliva Sample Plating" on the CyBio Felix; follow the on-screen instructions and prompts.

6.7. Seal the 384-well plate with an adhesive plate seal, place into a centrifuge, and spin briefly. Store at 4°C until the qPCR run is ready to begin.

7.0 <u>qPCR Procedure</u>

7.1. Check IntelliQube maintenance log, and perform daily / weekly / monthly maintenance, as needed. See TD-0005 for more information.

7.2. Access the LIMS on workstation 1, download the .csv sample file generated by the LIMS, which corresponds to each 384-well plate to be run.

7.3. Place an *Assay Plate* barcode label A-XXXXX on a LGC 96-well 0.8 mL storage plate. Create the run program in the *IntelliQube software* [TD-0005] and print the final plate layout and required assay mix volumes.

7.4. Ensure the IntelliQube has enough assay tape, tape seals, DI water, and bleach for the run.

7.5. Load the labeled *Assay Plate* with the assay mix as per the plate layout.

7.6. Load the 384-well sample plates into the *IntelliQube sample rack hotel* and place the *Assay Plate* in *Position 1* on the *IntelliQube*. Use the *IntelliQube Interface* to select the appropriate protocol and start the run [TD-0005].

8.0 Results and Reporting

Results from the IntelliQube are automatically sent to the LIMS software and Ugentec's FastFinder software (refer to TD-0009), where calls are generated according to the following decision tree:

PC	ROX	N1N2	RP	Result	Action
-	ANY	ANY	ANY	Retest	Retest
+	-	ANY	ANY	Retest	Retest
+	+	+	+	Positive	Report
+	+	+	- or Inc.	Inconclusive	Report
+	+	Inc.	ANY	Inconclusive	Report
+	+	-	+	Negative	Report
+	+	-	- or Inc.	Invalid	Report

The testing lab will be able to access the raw data through FastFinder and the LIMS. The clinical lab scientist (CLS) will review the results in FastFinder to and approve the run. After data authorization, the results will be reconciled with the sample manifest, uploaded to the LIMS, and made available for release to the medical provider.

9.0 Retests and Sample Storage/Retention

Invalid results require collection of a new specimen. A list of positive samples and those needing re-testing will be generated. At least once a day, samples needing re-testing will be pulled and re-run. Positive samples will be re-racked and stored at $\leq$ -20°C indefinitely. After these samples are removed, racks will be moved to post-processing storage and stored at 4°C for two weeks.

10.0 Limitations

10.1. Inadequate amount of sample provided (< 500 µL).
10.2. Improper collection of sample.
10.3. Viral load is below assay limit of detection (15 copies/µL raw saliva; 6 copies per reaction).
10.4. Presence of unidentified inhibitors.

11.0 Associated Documents

TD-0002 - FluidX Perception™ HD Plate Scanner Technical Document

TD-0003 - Operation of FluidX IntelliXcap 96-Tube Decapper

TD-0004 - Operation of Integra ViaFlo and Assist Plus

TD-0005 - IntelliQube Technical Document

TD-0006 - Operating a Water bath

TD-0007 – LIMS Technical Document

TD-0008 – Operation of the ViaFlo96

TD-0009 – Fastfinder software by Ugentec

TD-0010 – Operation of the CyBio Felix

REVISION HISTORY

Version Number	Summary of Changes in Current Version	Effective Date
SM-0001.01	New Document	12/03/20
SM-0002.01	Superplex Protocol	1/1/21

Addendum

Based on research and FastFinder SARS-CoV-2 result data analysis performed in-house, the consensus decision in regard to target results is as follows:

- N1N2 of Cq value below 33.00 will be marked as detected.

- N1N2 of Cq value equal to or above 33.00 will be marked as inconclusive.

- RNaseP of Cq value below 33.00 will be marked as detected.

- RNaseP of Cq value equal to or above 33.00 will be marked as not detected.

FastFinder will generate calls according to the decision tree, refer to 8.0. All invalid and inconclusive results will require collection of a new specimen.

Addendum 2

In an on-going effort to provide the best quality of SARS-CoV-2 RT-qPCR results, all positive samples have been routinely retested after reporting as part of quality assessment. Through this process, it was observed that not all calls with N1N2 Cq ≥31 were reproducible. To address this situation, all samples with N1N2 Cq ≥31 will be rerun within 24 hours prior to reporting. This supersedes the previous N1N2 "inconclusive" category (see Addendum from February 2021). The new reporting scheme for all samples with N1N2 Cq ≥31, but <40, is as follows:

Initial Run	Rerun	Final Result
N1N2 31≤Cq<40	N1N2 Cq<40	Detected
N1N2 31≤Cq<40	Not Detected or Cq>40	Inconclusive

Additionally, in rare cases where the fluorescence of a sample increases above baseline but does not have the normal sigmoidal form, the curve shape and Relative Fluorescence Unit reading can be used to interpret final results, which may differ from the above scheme as defined in Section 8.3 of TD-0009.02.

All inconclusive results will require collection of a new sample.

All samples with Cq <31 will be reported immediately after the initial run as described in the Standard Method.

1. <u>Objective</u>

The UC Davis High Throughput (UCD HTP) COVID-19 saliva test is intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in saliva samples. This method describes the procedure to prepare, analyze, and evaluate saliva samples for SARS-CoV-2 with multiplexed real-time reverse transcription PCR (rRT-PCR) on a high throughput instrument. For this "Superplex" protocol two fluorescent dyes are used for three probe-based assays in a single reaction. Two SARS-CoV-2 specific assays (the CDC designed nCOV-N1 and nCOV-N2 assays) use probes labeled with the same fluorescent dye (FAM). The human RNase P control assay (the CDC designed RNase P assay) employs probes labeled with a second dye (either HEX, QUA670, Atto-647, or Cal Fluor Orange [CFO]).

2. <u>Equipment, Reagents and Materials</u>

The following equipment and reagents are required. Equivalent equipment or reagents may be substituted.

2.1. LGC IntelliQube High-throughput qPCR system

2.2. FluidX IntelliXcap 96-tube Decapper, or equivalent

2.3. FluidX Perception™ HD Plate Scanner with IntelliCode software, or equivalent

2.4. Integra ViaFlow 12-channel automated pipette with Assist Plus robotic system, or equivalent

2.5. Integra ViaFlow96 96-channel automated pipette robotic system or Analytik-Jena Cybio Felix liquid handler robot, or equivalent

2.6. Integra 25 mL trough insert; PN #4311, #4312, or equivalent

2.7. Handheld 2D barcode scanner

2.8. 96-well 0.8 mL storage plate; LGC #KBS-7001-131, or equivalent

2.9. 384-well V-bottom plate; Greiner Bio-One #781280, or equivalent

2.10. qPCR master mix:

LGC RapiDxFire qPCR 5X Master Mix GF 10 mL; LGC #30050-2, and EpiScript RNase H Reverse Transcriptase 25,000 units; LGC #ERT12925K & ERT12925K-1.25ML *or* UltraPlex 1-Step ToughMix (4X); Quantabio #95166, or equivalent

2.11. LGC Biosearch primers and probes (2019-nCoV CDC-qualified Probe and Primer Kits for SARS-CoV-2) or their equivalents (e.g. IDT SARS-CoV-2 [2019-nCoV] CDC qPCR Probe Assay):

2.11.1. 2019-nCov CDC Probe and Primer Kit for SAR-CoV-2; LGC #KIT-nCoV-PP1-1000

2.11.2. 2019-nCoV_N2 Reverse Primer - 1000 nmol (NCOV-N2-R-1000)

2.11.3. 2019-nCoV_N2 Forward Primer - 1000 nmol (NCOV-N2-F-1000)

2.11.4. 2019-nCoV_N2 Probe (FAM labeled) - 250 nmol (NCOV-N2-P-250)

2.11.5. Human RNase P Extraction Control Reverse Primer - 1000 nmol (RNP-R-1000)

2.11.6. Human RNase P Extraction Control Forward Primer - 1000 nmol (RNP-F-1000)

2.11.7. Human RNase P Extraction Control Probe (QUA670 labeled) - 250 nmol (RNP-P-250)

2.11.8. 2019-nCoV_N1 Reverse Primer - 1000 nmol (NCOV-N1-R-1000)

2.11.9. 2019-nCoV_N1 Forward Primer - 1000 nmol (NCOV-N1-F-1000)

2.11.10. 2019-nCoV_N1 Probe (FAM labeled) - 250 nmol (NCOV-N1-P-250)

2.11.11. RNAse P oligos (Eurofins or equivalent): RNase P forward and reverse primers in 1X Low-EDTA TE Buffer (100 µM each) and RNase P probe - HEX-labeled in 1X Low-EDTA TE Buffer (100 µM), or equivalent (sequence identical to the LGC and IDT primers and probes above)

2.11.12. Premixed Superplex probe-set: N1N2RNP-RUO-1010 (CFO labeled)

2.12. BEI gamma irradiated SARS-CoV-2 virus positive controls; BEI Resources e.g. NR-52287

2.13. SuperROX® Reference Dye; Biosearch Technologies #SR-1000, or equivalent

2.14. 10X TBE buffer (Tris, Boric Acid, EDTA); Bio-RAD #1610733, or equivalent

2.15. TE buffer (10 mM Tris, 1 M EDTA, pH 8.0); Invitrogen #AM9849, or equivalent

2.16. Low-EDTA TE buffer (10 mM Tris, 0.1 M EDTA, pH 8.0); Teknova #T0223, or equivalent

2.17. Papain (powder, purified); Spectrum Chemical #PA125, or equivalent

2.18. RiboGuard RNase Inhibitor (40x); Biosearch Technologies #RG90910K, or equivalent

2.19. Water, RNase free, Molecular Biology Grade; Corning #46-000-CM, or equivalent

2.20. Computer, Windows 10, with Internet access and USB3 port

3. Preparation/Use of Reagents and Standards

Alternative volumes may be used, as long as the concentrations remain the same.

3.1. 1X TBE Solution

Add 100 mL 10X TBE to a clean empty liter bottle, and then fill to the 1 L mark with RNase-free water.

3.2. 5% Papain Solution

Weigh out 22.5 g Papain and transfer to a clean empty liter bottle. Fill to 350 mL with 10x TBE Solution. Allow Papain to dissolve, and then fill to 450 mL. This solution is stable for 7 days when stored at 4°C.

3.3. 80X Superplex Primer and Probes

3.3.1. Using the 2019-nCoV Primer and Probe kit:

3.3.1.1. The lyophilized 2019-nCoV Primer and Probe come in pre-aliquoted microfuge tubes. <u>Discard the RNase P primer and probe mix tube.</u> Allow the N1 and N2 tubes to reach ambient temperature, and then briefly centrifuge using a microcentrifuge. Resuspend the N1 and N2 Primer and Probe mix by adding 90 µL 1X Low-EDTA TE Buffer and vortexing briefly to dissolve. Centrifuge to collect all liquid at the bottom of the tube. Wait at least 20 minutes and centrifuge again.

3.3.1.2. Label a tube "SP Assay 80X" and add the following:
90 µL LGC N1 primer/probe mix
90 µL LGC N2 primer/probe mix
25.2 µL RNase P-F primer (100 µM)
25.2 µL RNase P-R primer (100 µM)
25.2 µL RNase P HEX probe (100 µM)

3.3.2. Using individual primers and probes:

3.3.2.1. Making stock solutions: Allow lyophilized tubes to come to ambient temperature, and then briefly centrifuge using a microcentrifuge. Resuspend all primers in 2.5 ml 1X Low-EDTA TE to 400 µM and probes in 1 ml 1X Low-EDTA TE Buffer to 250 µM. Vortex briefly to dissolve, centrifuge to collect all liquid at the bottom of the tubes, wait at least 20 minutes, vortex and centrifuge again.

3.3.2.2. After resuspended, dilute the RNase P primers 4X and all probes 2.5X so each has a final concentration of 100 µM.

3.3.2.3. Label a tube "SP Assay 80X Stock" and add 25 µL of each:
N1 forward primer (400 µM)
N1 reverse primer (400 µM)
N1 probe (100 µM)
N2 forward primer (400 µM)
N2 reverse primer (400 µM)
N2 probe (100 µM)
RNase P forward primer (100 µM)
RNase P reverse primer (100 µM)
RNase P probe (100 µM)
1X Low-EDTA TE Buffer

3.3.2.4. Vortex the oligos, spin down and store at 4°C, protected from light, for up to a month.

3.4. Assay Mix

*** *Use the same day or freeze in aliquots, shielded from light. Freeze/thaw less than five times*** *

Make a mix of PCR reagents using RNase-free water, RapiDxFire qPCR 5X Master Mix GF, EpiScript RNase H Reverse Transcriptase, SuperROX, and Superplex 80X probe mix in a ratio of 0.515 : 0.400 : 0.050 : 0.010 : 0.025. If not using Superplex 80X probe mix, add primers and probes individually. Mix to combine.

or

Make a mix of PCR reagents using RNase-free water, QuantaBio UltraPlex Master Mix (4X), SuperRox (15 µM), and Superplex 80X probe mix in a 0.465: 0.500 : 0.010 : 0.025 ratio. Mix to combine.

3.5. Use of Equivalent Reagents

Validation of Superplex Assay requires the use of an alternative RNase P probe with HEX dye to differentiate from the FAM dye used in the 2019-nCoV CDC nCoV-2 Probe and Primer kit. Probes and primers listed in section 2 contain the same sequence as the CDC nCoV-2 Probe and Primer kit and post analytical validation can be used.

4. Test Controls

Positive Control (COVP1):	BEI γ-irradiated virus controls diluted in TE buffer/1x Riboguard (or equivalent) to 10,000 copies per µL, then added to negative human saliva samples to a final concentration of at least 250 copies per µL
Negative Control (No Template Control [NTC]):	1X TBE buffer
Internal Process Control (IPC):	Human RNase P
Passive Reference Control:	Super-ROX dye in every reaction well

5. Sample Receiving Procedure

5.1. Samples arrive in 1 mL screw-top tubes in a 96-tube SBS format rack.

5.2. Place sample rack(s) in a 80-85°C water bath for 15 min. Place no more than four racks in a water bath at a time. If more than four racks are received, keep the remaining racks in the plastic bin labeled "Raw, non-deactivated saliva" until they are able to be processed. Refer to TD-0006.

5.3. After 15 min. remove sample rack(s) from the water bath and place in a pre-processing bin. Spin upside down in the manual centrifuge to remove fluid from the

sample tube caps. Then spin right side up to remove the remaining water out of the rack, and the saliva away from the inside of the tube caps. Remove the rack lids and allow cooling to room temperature for approximately ten minutes and air dry.

5.4. Scan sample tubes with a *FluidX Perception HD plate scanner* as outlined in TD-0002, and register sample information in the LIMS software as described in TD-0007.

5.5. Remove the rack lid and remove the sample tube caps using a *FluidX IntelliXcap* decapper, or equivalent.

5.6. Add 30 µL Papain to each tube of a rack using one of the following methods.
   5.6.1. *Integra Assist Plus*
      5.6.1.1. Place the sample rack in *Position B*.
      5.6.1.2. Load a rack of 300 µL pipette tips onto the *Assist Plus Position 1* and a *FluidX 25 mL fluid trough* into *Position A*. Add approximately 5 mL of 5% Papain solution, plus 3 mL per 96-tube sample rack, to the trough. For instance, four racks would require 17 mL of 5% Papain solution. Greater volumes may be used, as the numbers listed above are a minimum. Refer to TD-0004.
      5.6.1.3. Select the "Papain NT Singl" program on the *Integra ViaFlow Pipette*. Refer to TD-0004 for assistance using the *ViaFlow Pipette*. This program will add 30 µL of 5% Papain solution to all sample tubes in *Position B* on the *Assist Plus*.
   5.6.2. Once the program is complete, transfer the sample rack back to the *FluidX IntelliXcap*, re-cap the sample tubes, and replace the rack lid.
   5.6.3. Alternatively, carry out the Papain to saliva samples addition using the CyBio Felix liquid handling robot; follow the on-screen instructions and prompts (refer to TD-0010).

5.7. Place rack(s) in storage at 4°C for later use, or proceed to step 5.9

5.8. For samples stored at 4°C, remove sample rack(s) from the refrigerator. Space racks apart from each other and allow to sit at room temperature for a minimum of 30 minutes, but no longer than 2 hours.

5.9. For Papain digestion, place racks on either Shaker 1 or Shaker 2 (maximum 8 racks per shaker), start a timer for 30 minutes, and turn on Shaker to mix. When five minute mixing program is complete, allow samples to sit for the remainder of the 30 minutes at room temperature.

5.10. After the 30 minute incubation is complete, place sample rack(s) in a 95 ± 3°C water bath for 20 minutes. Refer to TD-0006. Then remove samples from the water bath and allow them to cool to room temperature and air dry.

6. Sample Preparation Procedure

Determine the number of sample racks to be analyzed (1-4) and the priority samples needing testing. Reserve a barcoded 384-well plate and use this plate barcode as the Batch ID.

6.1. Organize the racks numerically by barcode, and assign them numbers 1-4. Then reorganize samples leaving an empty space in each rack for controls, as follows:

6.1.1. rack 1: A2

6.1.2. rack 2: A11

6.1.3. rack 3: H2

6.1.4. rack 4: H11

6.2. Rerack samples to include priority samples and consolidate racks to 95 samples each. If there is not enough space for all priority samples, move some of the original samples to the priority rack to be processed in the next run.

6.3. The rearranged racks now have the samples in their final positions, with empty spaces for the controls.

6.4. Scan the sample racks with the *FluidX Perception HD plate scanner* to record the final tube positions and create the 384-well plate in the LIMS. Have a second person verify the 384-well plate was correctly created in the LIMS.

6.5. Load 1X TBE into 384-well plate (Integra Assist Plus)

6.5.1. Place the clean, barcoded 384-well plate, selected in step 6.0, into *Position C* on the *Assist Plus*, and place a clean 25 mL trough in *Position A*.

6.5.2. Load a rack of 300 μL pipette tips into Position 1.

6.5.3. Add at least 17 mL of 1X TBE, to the trough.

6.5.4. Transfer 25 μL of 1X TBE to the 384-well plate. Refer to TD-0004.

6.5.5. Alternatively, carry out the 1X TBE pre-loading with the program "384W TBE Buffer Prefill" on the CyBio Felix; follow the on-screen instructions and prompts.

6.6. Transfer the saliva to the 384-well plate

6.6.1. Decap the tubes, and for rack 1 add the positive control COVP1 tube to position A2 in the rack.

6.6.2. Have a second person verify the rack barcode, controls, and the position/orientation of each rack on the instrument used to create the 384-well plate.

6.6.3. Transfer 25 µL of sample from each of the rack(s) to the 384-well plate using CyBio Felix and follow the on-screen instructions and prompts (see TD-0010).

6.6.4. Alternatively, the Integra Viaflow 96 may be used as a backup, if needed.

6.7. Seal the 384-well plate with an adhesive plate seal, place into a centrifuge, and spin briefly. Store at RT until the qPCR run is ready to begin. If plate needs to be stored for longer than 4 hours, place in -20°C freezer.

7. <u>qPCR Procedure</u>

7.1. Check IntelliQube maintenance log, and perform daily / weekly / monthly maintenance, as needed. See TD-0005 for more information.

7.2. Access the LIMS on workstation 1 or laptop connected to Genome Center network and download the .csv sample file generated by the LIMS, which corresponds to each 384-well plate to be run.

7.3. Place an *Assay Plate* barcode label A-XXXXX on a LGC 96-well 0.8 mL storage plate. Create the run program in the *IntelliQube software* (refer to TD-0005) and print the final plate layout and required assay mix volumes.

7.4. Ensure the IntelliQube has enough assay tape, tape seals, DI water, and bleach for the run.

7.5. Load the labeled *Assay Plate* with the assay mix as per the plate layout.

7.6. Load the 384-well sample plates into the *IntelliQube sample rack hotel* and place the *Assay Plate* in *Position 1* on the *IntelliQube*. Use the *IntelliQube Interface* to select the appropriate protocol and start the run (refer to TD-0005).

8. Results and Reporting

8.1. Results from the IntelliQube are automatically sent to the LIMS software and Ugentec's FastFinder software (refer to TD-0009), where calls are generated according to the following decision tree:

PC	ROX	N1N2	RP	Result	Action
-	ANY	ANY	ANY	Retest	Retest
+	-	ANY	ANY	Retest	Retest
+	+	+	+	Positive	Report
+	+	+	- or Inc.	Inconclusive	Report
+	+	Inc.	ANY	Inconclusive	Report
+	+	-	+	Negative	Report
+	+	-	- or Inc.	Invalid	Report

8.2. The following applies to RNase P target resulting:

8.2.1. RNase P of Cq value < 33.00 will be marked as **detected.**

8.2.2. RNase P of Cq value $\geq$ 33.00 will be marked as **not detected.**

8.3. All samples with N1N2 Cq ≥31 will be rerun within 24 hours prior to reporting. The reporting scheme for all samples with N1N2 Cq ≥31, but <40, is as follows:

Initial Run	Rerun	Final Result
N1N2 31≤Cq<40	N1N2 Cq<40	**Detected**
N1N2 31≤Cq<40	Not Detected or Cq>40	**Inconclusive**

8.3.1. Additionally, in rare cases where the fluorescence of a sample increases above baseline but does not have the normal sigmoidal form, the curve shape and Relative Fluorescence Unit (RFU) reading can be used to interpret final results, which may differ from the above scheme, as defined in Section 8.3 of TD-0009.02.

8.3.2. All samples with Cq <31 will be reported immediately after the initial run.

8.4. The testing lab will be able to access the raw data through FastFinder and the LIMS. The clinical lab scientist (CLS) will review the results in FastFinder to approve the run. After data authorization, the results will be reconciled with the sample manifest, uploaded to the LIMS, and made available for release to the medical provider.

9. **Retests and Sample Storage/Retention**

   9.1. Invalid and inconclusive results require collection of a new specimen.

   9.2. A list of positive samples and those needing re-testing will be generated. At least once a day, samples needing re-testing will be pulled and samples re-run.

   9.3. Positive samples will be re-racked and stored at $\leq$ -20°C indefinitely.

   9.4. After positive samples are removed, racks will be moved to post-processing storage and stored at 4°C for two weeks.

10. **Limitations**

    10.1. Inadequate amount of sample provided (< 500 µL)

    10.2. Improper collection of sample

    10.3. Viral load is below assay limit of detection (12.5 copies/µL raw saliva; 5 copies per reaction)

    10.4. Presence of unidentified inhibitors.

11. **Associated Documents**

    TD-0002 - FluidX Perception™ HD Plate Scanner Technical Document
    TD-0003 - Operation of FluidX IntelliXcap 96-Tube Decapper
    TD-0004 - Operation of Integra ViaFlo and Assist Plus
    TD-0005 - IntelliQube Technical Document
    TD-0006 - Operating a Water bath
    TD-0007 - LIMS Technical Document
    TD-0008 - Operation of the ViaFlo96
    TD-0009 - FastFinder software by Ugentec
    TD-0010 - Operation of the CyBio Felix

REVISION HISTORY

Version Number	Summary of Changes in Current Version	Effective Date
SM-0002.02	Superplex Protocol	6/11/21