1. PURPOSE

1.1 To verify the LOD for the molecular diagnostic assay that utilizes real-time polymerase chain reaction (PCR) in order to determine the presences or absences of SARS-CoV-2 RNA in human nasal swabs at CDPH Branch Laboratory, Valencia, CA.

2. SCOPE

2.1 This procedure applies to individuals performing the SARS-CoV-2 Assay to be performed at CDPH Branch Laboratory, Valencia, CA.

2.2 This validation report is for SARS-CoV-2 RT-qPCR Reagent kit (Cat # 2019-nCoV-PCR-AUS).

2.2.1 These tests are completed using contrived samples created by spiking encapsulated positive control RNAs for SARS-CoV-2 *ORF1ab* and *N* gene using Positive Reference Material from AccuPlexTM SARS-CoV-2 Molecular Controls Kit – Seracare, Full Genome, Cat# 0505-0159, Part# 0505-0163 (Certificate of Analysis must be obtained to determine stated copies/mL per lot), into VTM and Inactivating VTM.

3. DEFINITION

3.1 IC: An exogenous internal control comprised of TE buffer and bacteriophage MS2, provided in the kit (Cat #: 2019-nCoV-PCR-AUS, tube: nCoV Positive Control)

3.2 Ct or Cq: Cycle number at which PCR response starts to become exponential

3.3 AN: Anterior Nasal

3.4 NSP: Nasopharyngeal

3.5 *ORF1ab* and *N* genes: 2 different coding areas in the SARS-CoV2 genome interrogated in this validation using RT-PCR

3.6 *RPP30 (encodes RNAseP)- a human gene target included in some assays to verify adequacy of specimen collection, human origin of specimen, and as a second extraction control.*

3.7 FAM, ROX, HEX/VIC: Abbreviations for 3 fluorescent dyes used in reporter oligonucleotides in RT-PCR assay. FAM probe reports on SARS-Cov2 *N* gene, ROX on *ORF1ab* gene, and HEX reports on the IC (bacteriophage MS2)

3.8 RT-PCR: real-time PCR

3.9 SARS-CoV-2: Severe acute respiratory syndrome-related coronavirus of the genus *Betacoronavirus*, strain 2

3.10 Viral Transport Medium (VTM)/Molecular Transport Medium (MTM): synthetic meda in which human nasal swabs are transported to testing labs. In this validation, two types of VTM/MTM will be utilized:

3.10.1 Viral Transport Media (VTM) (Cat# 600-102)

3.10.2 PrimeStore MTM (Cat# MT0501-1 & MT0501-2)

4. ROLES AND RESPONSIBILITIES

4.1 Responsibilities

4.1.1 It is the responsibility of the laboratory and delegates to establish any methods required to perform this assay. This must be properly documented in a validation plan, report, standard operating procedures, and any other required documentation.

4.1.2 All personnel that will perform this assay must understand and comply with established procedures.

4.1.3 CDPH Branch Laboratory management will provide leadership and support to ensure the development of a properly validated assay in a safe working environment.

4.1.4 The Laboratory Director will approve the validation plan and report. This approval will authorize the test for clinical use.

4.2 Key Personnel

4.2.1 Clinical laboratory staff at CDPH Branch Laboratory Valencia, CA

4.2.2 Adam Rosendorff, MD, Laboratory Director

5. BACKGROUND / CLINICAL SIGNIFICANCE

5.1 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, previously called 2019-nCoV) is a beta-coronavirus, a novel coronavirus belonging to a family of coronaviruses.

5.2 The PerkinElmer® New Coronavirus Nucleic Acid Detection Kit is a real-time RT-PCR *in vitro* diagnostic test intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in human specimens collected from individuals suspected of SARS-CoV-2 by their healthcare provider.

5.3 Results are for the identification of SARS-CoV-2 RNA, which is generally detectable in human respiratory specimens during the acute phase of infection While positive results indicate 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.[11.1]

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

6. PROTOCOLS AND EQUIPMENT TO BE USED IN THIS VALIDATION

6.1 Controlled Document Titles and Reference ID that will be used in this validation:

6.1.1 Accessioning for SARS-CoV-2 Samples (CA-ACC-SOP-001)

6.1.2 Heat Inactivation of Viral Swab Samples (CA-EXT-SOP-001)

6.1.3 Decapping and Batch Preparation for Janus G3 (CA-EXT-SOP-002)

6.1.4 Sample Transfer Using the Janus G3 (CA-EXT-SOP-003)

6.1.5 Viral RNA_DNA Extraction Using the chemagic™ 360-D (CA-EXT-SOP-004)

6.1.6 SARS-CoV-2 RT-PCR Set-up Using Janus G3 (CA-PCR-SOP-001)

6.1.7 SARS-CoV-2 RT-PCR Using the Analytik Jena (CA-PCR-SOP-002)

6.1.8 SARS-CoV-2 Assay Data Extraction (CA-RPT-SOP-001)

6.1.9 Analysis and Reporting of SARS-CoV-2 Assay (CA-RPT-SOP-002)

6.2 Equipment and Systems with ID

Table A. Equipment and System IDs

Equipment	Manufacturer	Model	Part/Cat#	Purpose	Environmental Requirements Temperature/ Humidity
chemagic™	PerkinElmer®	360 or 360-D	20240056	DNA/RNA Extraction	Temp: 18 - 35 °C Humidity: < 80 %
Biosafety Hood	Any	Any	Any	Prepare master mix	Dependent on model
JANUS G3	PerkinElmer®	Any	Any	Sample Reformatting and PCR workstation	Temp: 15 – 35 °C Humidity: 60 – 80 %
Microcentrifuge	Any	Any	Any	Collect samples at bottom of well	Dependent on model
Vortex mixer	Any	Any	Any	Mix reagents and/or samples	Dependent on model
Plate Centrifuge	Any	Any	Any	Collect samples at bottom of well	Dependent on model

Table A. Equipment and System IDs					
Equipment	**Manufacturer**	**Model**	**Part/Cat#**	**Purpose**	**Environmental Requirements Temperature/ Humidity**
Pipettes (single and multi-channel) p10, p200, p1000	Any	Any	Any	Pipette reagent and samples	Dependent on model
Heratherm Oven	Thermo Scientific	Any	Any	Heat samples to kill virus	Temp: 15 - 31 °C Humidity: 10 - 85 %
qPCR Real-Time System	Analytica Jena	AJ384	844-00569-4	RT-PCR	Temp: 15 - 31 °C Humidity: 10 - 85 %

6.3 Reagents, Supplies, Materials, etc.

Table B. Reagents					
Reagent	**Vendor**	**Manufacturer**	**Part/ Cat#**	**Purpose**	**Storage Conditions**
chemagic™ Viral 300 RNA/DNA Kit H96 component Magnetic Beads B 1 bottle, 250 mL Prep: Ready to use Exp: Unopened, according to the labeled Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Bind RNA/DNA	2 - 25 °C
chemagic™ Viral 300 RNA/DNA Kit H96 component Lysis Buffer 1 1 bottle, 500 mL Prep: Ready to use Exp: Unopened, according to the label Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Lyses cells or other DNA source to get the RNA/DNA into solution	2 - 25 °C

Table B. Reagents					
Reagent	**Vendor**	**Manufacturer**	**Part/ Cat#**	**Purpose**	**Storage Conditions**
chemagic™ Viral 300 RNA/DNA Kit H96 component Binding Buffer 2 1 bottles, 2.5 L Prep: Ready to use Exp: Unopened, according to the label Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Create condition to allow the RNA/DNA to bind to the Magnetic beads	2 - 25 °C
chemagic™ Viral 300 RNA/DNA Kit H96 component Wash Buffer 3 1 bottle, 1 L Prep: Ready to use Exp: Unopened, according to the label Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Remove non-DNA/RNA contaminants during washing step	2 - 25 °C
chemagic™ Viral300 RNA/DNA Kit H96 component Wash Buffer 4 1 bottle, 1 L Prep: Ready to use Exp: Unopened, according to the label Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Remove non-DNA/RNA contaminants during washing step	2 - 25 °C
chemagic™ Viral 300 RNA/DNA Kit H96 component Wash Buffer 5 1 bottle, 1 L Prep: Ready to use Exp: Unopened, according to the label Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Remove last traces of non-DNA/RNA contaminates	2 - 25 °C

Table B. Reagents					
Reagent	**Vendor**	**Manufacturer**	**Part/ Cat#**	**Purpose**	**Storage Conditions**
chemagic™ Viral 300 RNA/DNA Kit H96 component Elution Buffer 6 1 bottle, 250 mL TRIS-HCl pH 7.8 - 8.4 Prep: Ready to use Exp: Unopened, according to the label Opened: 30 days	PerkinElmer®	AUS	CMG-1033-S	Elute DNA/RNA	2 - 25 °C
chemagic™ Viral 300 RNA/DNA Kit H96 component Proteinase K 1 * 11 mL bottle Prep: Reconstituted with 11 mL molecular grade water Exp: Unopened, according to the label Opened: 14 days, 2 - 8 °C	PerkinElmer®	AUS	CMG-1033-S	Added to enhance the efficiency of the lysis step	2 - 8 °C
Molecular Biology Grade Water	Any	Any	Any	Used to reconstitute Proteinase K, NTC control	15 - 25 °C
chemagic™ Viral 300 RNA/DNA Kit H96 component Poly (A) RNA 10 bottle Prep: Reconstituted with 440 µL of Poly (A) RNA Buffer Mix Exp: Unopened, according to label Opened: 4 weeks, 2 - 8 °C	PerkinElmer®	AUS	CMG-1033-S	Added to enhance the efficiency of the lysis step	2 - 8 °C

Table B. Reagents					
Reagent	**Vendor**	**Manufacturer**	**Part/ Cat#**	**Purpose**	**Storage Conditions**
chemagic™ Viral 300 RNA/DNA Kit H96 component Poly (A) RNA Buffer Mix 10 bottle, 2 mL Exp: Unopened, according to label Opened: 30 days.	PerkinElmer®	AUS	CMG-1033-S	Added to Poly (A) RNA to activate.	15 - 25 °C
Viral Transport media (VTM)	Approved vendors	GeminiBio	600-103	Media to create contrived LOD samples	15 - 25 °C
Viral Transport Medium (Inactivated) – Inactivating VTM Tris-HCl buffer solution	Yocon	Yocon Biology Technology Company	MT0501-1 & MT0501-2	Media to create contrived LOD samples	15 - 25 °C
AccuPlexTM SARS-CoV-2 Molecular Controls Kit – Full Genome	SeraCare	SeraCare Life Science, Inc.	0505-0159	Used to create contrived samples	2 - 8 °C
New Coronavirus Nucleic Acid Detection Kit	PerkinElmer®	PerkinElmer®	2019-nCoV-PCR-AUS	RT-PCR kit	-25 to -15 °C

Table C. Materials and Supplies					
Material and Supplies	**Vendor**	**Manufacturer**	**Part/Cat #**	**Purpose**	**Storage Conditions**
2 mL Deep Well Plate	PerkinElmer®	PerkinElmer®	CMG-555	Load sample/lysis and Elution Buffer B onto the chemagic™ 360	15 - 25 °C
96 Rod Head Disposable Tips	PerkinElmer®	PerkinElmer®	49017-0006	Protect 96 Rod Magnetic Head (on chemagic™ 360) from contamination	15 - 25 °C

Table C. Materials and Supplies					
Material and Supplies	**Vendor**	**Manufacturer**	**Part/Cat #**	**Purpose**	**Storage Conditions**
Low-well Plate	PerkinElmer®	PerkinElmer®	CMG-555-1	Load Magnetic Beads B onto the chemagic™ 360	15 - 25 °C
Heat Sealing Foil	Approved vendor	Approved vendor	Approved vendor	Seal plates	N/A
Reagent trough	Any	Any	Any	Preserve reagent stock bottle and allow for multi-channel pipetting	N/A
Tips (10 µL, 200 µL, 1000 µL) compatible with pipettes	Any	Any	Any	Pipette reagent and samples	Dependent on brand
384 Plates for RT-PCR	Approved vendor	Approved vendor	Approved vendor	Hold samples and master mix	N/A
qPCR Seal	Approved vendor	Approved vendor	Approved vendor	Seal 384 well plate	N/A; Expiration Date is written on the box
1.7 mL to 50 mL tube	Any	Any	Any	For master mix preparation	Dependent on brand

6.4 Training Requirements

6.4.1 Personnel that performing this assay will require proper training. This training will include complete review and understanding of all associated documentation and hands-on laboratory training in the performance of this assay.

7. SPECIMENS REQUIRED

7.1 Negative control: nCoV negative control from 2019-nCoV-PCR-AUS Kit, run in every extraction plate

7.2 Positive control: nCoV positive control from 2019-nCoV-PCR-AUS Kit containing 1000 copies/mL of SARS-CoV-2 RNA fragments capsulated in bacteriophage, run in every extraction plate

7.3 Clinical samples: Samples previously referred for clinical testing.

7.4 Pooled negative samples: A sample created by combining samples previously tested and resulted as negative to obtain sufficient volume to perform reproducibility studies.

7.5 Pooled positive samples: A sample created by combining samples previously tested and resulted as positive to obtain sufficient volume to perform reproducibility studies.

7.6 Contrived samples: Samples created by spiking encapsulated positive control RNAs for SARS-CoV-2 *ORF1ab* and *N* gene using Positive Reference Material from AccuPlexTM SARS-CoV-2 Molecular Controls Kit – Full Genome, Cat# 0505-0159, Part# 0505-0163 (Certificate of Analysis must be obtained to determine stated copies/mL per lot), into viral media (VTM or MTM) OR pooled negative samples.

8. VALIDATION PROTOCOL

8.1 The SARS-CoV2 assay will be performed according to the protocols listed in Section 6.

8.1.1 Samples will be subjected to heat inactivation at 65^{o}C for 30 min (Convection oven). Samples will then be cooled at room temperature for at least 5 minutes, and briefly centrifuged to collect condensation at the bottom of the well at 1200 RPM for 1 minute.

8.1.2 Samples will be prepared for extraction by adding the following to a deep-well plate: 19 mL master mix consisting of Proteinase K (10 mL), poly A RNA (4 mL) and Internal Control (5 mL), followed by 300 mL of sample, and lastly 300 mL of Lysis Buffer.

8.1.3 RNA from all samples will be extracted using the chemagic™ 360 running the "chemagic viral300 360 H96 drying prefilling VD141210.che" program and using the chemagic™ Viral 300 RNA/DNA Kit H96 kit.

8.1.4 RT-PCR Setup

8.1.4.1 SARS-CoV-2 RT-qPCR reagent Kit: 10 mL of each eluate will be mixed with 5 mL of a real time PCR master mix prepared using tubes nCoV Reagent A, nCoV Reagent B, and nCoV Enzyme Mix.

8.1.4.2 The reactions will be subjected to RT-PCR in an AnalytikJena 384.

8.1.5 Quality Control and Data Analysis

8.1.5.1 Quality control and data analysis will be performed in accordance with kit instructions for the RT-PCR kit (Cat#: 2019-nCoV-PCR-AUS).

8.2 Protocol variation

8.2.1 Barcodes will be created specifically for this study.

8.2.1.1 Barcodes will be tracked in LIMC.

8.2.1.2 No data will be transferred outside of the laboratory for reporting.

8.2.2 Some studies in this validation will deviate from the established protocols such as the assay being performed with no heat inactivation or with the addition of primers for the *RPP30* target.

8.2.3 All protocol deviations with will be described in relevant sections of the plan.

8.2.4 Studies described in this plan may use automation or manual set-up options described the laboratory protocols.

9. VALIDATION PLAN

Table

Section	Study	Subsection	Sample type	Sample treatment	PCR Targets	Page
9.1	LoD Finding and Confirmation (media collection)	9.1.1	VTM	No heat	Viral + EC*	15
				Heat	Viral + EC	15
					Viral + EC+ *RPP30*	15
		9.1.2	MTM	No Heat	Viral + EC	15
				Heat	Viral + EC	15
				Heat	Viral + EC + *RPP30*	15
9.2	LoD Finding and Confirmation (dry collection)	9.2	Dry swab	N/A	Viral + EC + *RPP30*	17
9.3	Accuracy (Clinical Evaluation)	9.3.1	MTM	Heat	Viral + EC	20
					Viral + EC + *RPP30*	
		9.3.2	Dry Swab	N/A	Viral + EC + *RPP30*	21
9.4	Precision (Intra-run and Inter-run Reproducibility)	9.4.1	MTM	Heat	Viral + EC	23
					Viral + EC + *RPP30*	23
		9.4.2	Dry swab	N/A	Viral + EC + *RPP30*	23
9.5	Analytical Sensitivity (Inclusivity)	9.5.1	Bioinformatic data	N/A	N/A	25
9.6	Analytical Specificity (Cross-Reactivity)	9.6.1	Bioinformatic data	N/A	N/A	26
		9.6.2	Virus / pathogen panel in MTM	Heat	Viral + EC + *RPP30*	28
		9.6.3	Virus / pathogen panel in dry swab	Heat	Viral + EC + *RPP30*	28
9.7	Analytical Specificity (Interfering substances)	9.7.1	Various substances in MTM	Heat	Viral + EC + *RPP30*	30
						30
9.8	Sample stability	9.8.1	MTM sample	Heat	Viral + EC	32
					Viral + EC + *RPP30*	32
		9.8.2	Dry swab sample	N/A	Viral + EC + *RPP30*	32
		9.8.3	Extracted RNA	N/A	Viral + EC + *RPP30*	32

*Extraction control

9.1 Limit of Detection (LoD) Range Finding and Confirmation – Swabs in Media

9.1.1 Samples required: Contrived AccuPlexTM SARS-CoV-2 Molecular Controls Kit – Full Genome + Media

9.1.2 Study design

- Serial dilutions of AccuPlexTM SARS-CoV-2 Molecular Controls Kit – Full Genome in media indicated will be created according to Appendix A.
- Range Finding:
    - Dilutions to be tested are 10, 20, 40, 60, 120, 180 and 540 copies / mL
- Samples will be run in triplicate
- Samples will be run on laboratory protocols…
- The LoD will be established at the lowest concentration at which all three replicates are detected
- The LoD may be different for the *ORF1ab* and *N* genes.
- LOD confirmation:
    - LoD will be confirmed by running 20 replicates at:
    - The determined LoD
    - 0.3X of the LoD
    - 3X the LoD
- Detection of 19/20 replicates in LoD confirmation studies is required to confirm the LoD.

9.1.3 Study set-up

Set 1: VTM, no heat inactivation
Viral target + IC
Viral target + IC + *RPP30*
Set 2: VTM, heat inactivation
Viral target + IC
Viral target + IC + *RPP30*
Set 3: MTM, no heat inactivation
Viral target + IC
Viral target + IC + *RPP30*
Set 4: MTM, heat inactivation
Viral target + IC
Viral target + IC + *RPP30*

9.1.4 Data will be presented in Table 1a (finding) and Table b (confirmation) and the LoD for each gene will be stated in the report.

9.2 **LoD For Dry Swabs**

9.2.1 Dry Swab Validation

This is a qualitative assay approved for use under Emergency Use Authorization (EUA) and CLIA does not require validation of the Limit of detection (LOD). LOD studies were performed to confirm where the LOD lies. Based on results and speaking to other laboratories, the spiking of contrived material may not represent true patient LODs.

9.2.1.1 Verification of LOD and Precision

The LOD and precision will be evaluated in pools consisting of 5 specimens. Testing includes the individual positive specimens as well as 40 positive and 20 negative specimen pools. Each positive specimen pool consists of one positive specimen (negative specimen spiked with control material) with the remaining specimens being negative, whereas the negative specimen pools consisted only of negative specimens. The positive specimens used in the study should cover the lower limit of the detectable range of the assay (see Table below). Each pool is tested 4 times over a minimum of two days, two runs/day recommended.

Approximate Ct value (Positive Sample)	Number of pools
37-42	5
37-38	5
33-34	5
31-34	5
26-29	5
24-26	5
17-19	5
14-16	5

9.2.1.2 The validation data will be used to determine at what Ct value detection is lost by pooling. These data will be used to estimate the percentage of high Ct positive samples (Ct >37- $\leq$42) that are likely to be lost by pooling.

9.2.1.3 Precision will be defined as pools of samples tested in replicate giving the same result. 40 Positive samples/pools with a known Ct

value from singlicates testing will be included in precision studies.

9.2.1.4 A sample will be considered invalid if the internal control fails (as per instructions for use by each RT-PCR assay) or instrument error. (10.11)

9.2.1.5 Verification of Dry Swab Rehydration

Rehydration of dry swabs will be tested using known clinical positive and known clinical negative specimens. Each swab will be dipped in the well of the STO plate that has already gone through the extraction and PCR process. The swabs will be dried for 20 minutes at room temperature, then placed inside an empty collection tube. The tubes will then be placed into the Reformatter cassette and 1mL of 50% Lysis Buffer will be added to each of the tubes. The cassettes then then be vortexed for 30 seconds at medium strength, then incubated at room temperature for 30 minutes. The samples will then be processed as a singleton sample in normal downstream processes.

32 positive samples and 32 negative samples will be evaluated based on the concordance with their original individual result. The Ct value of the rehydrated dry swab samples will be compared to the original Ct value from the routine process to determine percent concordance.

9.2.1.6 Verification of Dry Swab Stability

Dry swab stability testing will provide evidence of how the quality of the RNA varies with time under the influence of a variety of environmental factors such as temperature and humidity.

Dry swab stability will be evaluated using 20 positive samples, in combinations of 5 each for 4 positive pools. 5 swabs will be dipped into each of the 4 pools and tested at different times. The first swab will be used as a baseline and run immediately while the other 4 will be tested at 12, 24, 72, 96 hours. The data will be evaluated based on the percent concordance between the different time periods.

9.2.1.7 Estimate at what Ct value we may lose detection by dry swab rehydration. Compare this this to original Ct values, what percentage of high Ct positive samples are we likely to lost by dry

swab rehydration?

9.2.2 **ACCEPTANCE CRITERIA AND EXPECTED RESULTS**- All clinical samples should be positive for the internal control, otherwise marked invalid and not included in the analysis.

- 27/30 (9%) of pooled samples with Ct of 34 or lower should be detected on the first day.

- Evaluation of Ct values in individual sample results will be compared to the same positive patient pooled samples. 3/30 (10%) of singletons with Ct below 35 will not be detected in a rehydrated dry swab samples; Cts above 35 are not expected to be detected.

-90% of positive rehydrated samples should have positive Ct values for both FAM and ROX.

- *RPP30* should be present in each sample with a Ct value less than 35 (acceptance 29/30 (~97%).

9.3 Accuracy (Clinical Evaluation)

9.3.1 Samples required: Previously tested nasopharyngeal clinical samples

- Information about the assay used clinically, the LoD of the comparator assay, media used for collection, as well as the outcome (positive or negative, including Ct values for the *N* and *ORF1ab* genes) will be obtained.
- Positive samples with both high (Ct >37- ≤42) and low Ct (Ct ≤37) values will be used
- Samples not originally collected in MTM media will be spiked 1:1 with clean MTM.
- Previously tested samples will be obtained from the California Department of Public Health Laboratories (Dr. Paul Kimsey) and from The Department of Pathology, Medical College of Georgia (Dr. Ravindra Kolhe)
- Information about the assay used clinically, the LoD of the comparator assay, media used for collection, as well as the outcome (positive or negative, including Ct values for the *N* and *ORF1ab* genes) will be obtained.
- The comparator assay should have an LoD comparable to the PerkinElmer New Coronavirus Kit (~120 copies/mL)
- A minimum of 90 samples will be run:

Clinical Classification based on Comparator Assay	**Samples**
SARS-CoV-2 Not Detected	30
SARS-CoV-2 Detected (High Ct*)	30
SARS-CoV-2 Detected (Low Ct*)	30

*High Ct - defined as Ct > 37 and ≤ 42, Low Ct defined as Ct <37

9.3.2 Study design

- Samples not originally collected in MTM media will be spiked 1:1 with clean MTM.
- PCR will be performed with and without primers for *RPP30*
- A minimum of 90 samples will be run:

Clinical Classification based on Comparator Assay	**Samples**	**Number Detected by SARS-CoV-2 RT-qPCR Reagent Kit**				
		-RPP30		***+RPP30***		
		N	***ORF1ab***	***N***	***ORF1ab***	***RPP30***
SARS-CoV-2 Not Detected	30					
SARS-CoV-2 Detected (High Ct*)	30					
SARS-CoV-2 Detected (Low Ct*)	30					

*High Ct - defined as Ct > 37 and ≤ 42, Low Ct defined as Ct <37

9.3.3 Dry Swab Samples Clinical Evaluation

9.3.3.1 Verification of *RPP30* (human gene) detection

RNA extracted from dry swabs will be tested with and without RPP30 to determine a baseline for home collection. Swabs will be dipped into thirty-two extracted positive and thirty-one extracted negative samples and will be run on The New Coronavirus Nucleic Acid Detection kit. A second run using the same samples with be run on The New Coronavirus Nucleic Acid Detection kit with added *RPP30*. The *RPP30* gene detection will be evaluated by the presence of the gene in the sample. Ct values will also be evaluated to determine whether the addition of *RPP30* affects the LoD using The New Coronavirus Nucleic Acid Detection kit.

9.3.3.2 A sample will be considered invalid if the internal control fails (as per instructions for use by each RT-PCR assay) or instrument error.

9.3.4 Interpretation

- Samples with IC drop-out in a negative sample will be excluded from the analysis.
- Positive and negative controls must be valid
- Results will be compared to comparator sample key.
    - Positive samples are expected to be detected
    - Negative samples are expected to be not detected
- The following exception are possible:
    - Apparent false positives: High Ct values detected by this assay may reflect a false negative result in the comparator assay. This is expected due to the high sensitivity of the assay being tested.
    - Apparent false negatives: High Ct values detected in the

comparator assay may test negative if the sample integrity has been compromised by time or freeze-thaw cycles. These exceptions are expected to be rare and unpredictable.

9.3.5 Acceptance criteria:

- ≥ 98% positive predictive agreement
- ≥ 98% negative predictive agreement

9.4 Precision (Intra-run and Inter-run Reproducibility)

9.4.1 Samples required: samples run in-house at the CDPH Branch Laboratory, Valencia to create 3 pooled samples

- Negative samples: ~30
- High positive samples (Ct values >37 - ≤ 42): ~30
- Low positive samples (Ct values ≤ 37): ~30

9.4.2 Study design

Intra-run (same day):

- Extract each pool (negative, high positive and low positive) 10 times (30 extraction total)
- Perform SARS-CoV2 assay on all extractions (30 total)
- Repeat assay later in the same day (30 total)

Inter-run (across three days):

- Extract each pool (negative, high positive and low positive) 10 times (30 extraction total) on each of three days
- Perform SARS-CoV2 assay on all extractions (30 total)
- Repeat assay on three different days by different technologists.

9.4.3 Verification of Intra-run Precision for Dry Swabs

9.4.3.1 Intra-run precision is the variability in the data from replicate determinations of the same homogenous sample used in a method or procedure. It is expected that the same sample will give similar results.

9.4.3.2 Intra-run precision will be determined by running 15 positive samples and 85 negative samples over two days. The data will be evaluated based on the percent concordance between the two runs.

9.4.3.3 From the data, the Ct value at which detection is lost due to dry swab rehydration will be determined. It is expected that Ct values greater than 35 in routine testing will be not detected due to the dry swab process. These data will be used to estimate the percentage of high Ct positive samples that are likely to be lost by dry swab rehydration.

9.4.3.4 A sample will be considered invalid if the internal control fails (as per instructions for use by each RT-PCR assay) or instrument error.

9.4.4 Interpretation

- Samples with IC drop-out in a negative sample will be excluded from the analysis.
- Positive and negative controls must be valid
- Positive samples are expected to be detected
- Negative samples are expected to be not detected

9.4.5 Acceptance Criteria

- 10/10 negative samples will be not detected in each run
- 10/10 low Ct samples will be detected
- 6/10 high Ct samples will be detected in each run

9.5 Analytical Sensitivity (Inclusivity)

9.5.1 *In silico* Analysis

- SARS-CoV2-2 specific primers and probes used in this assay will be compared to the NCBI and GISAID databases
- Inclusion criteria for sequences used for comparison will be:
    - Complete sequences: Genomes with >29000bp
    - High coverage inclusivity: Entries with <1% N base pair and <0.05% unique nucleic acid substitutions (not seen in other sequences in the database) and no indels unless verified by the submitter
    - High coverage: Entries with >5% N base pairs were excluded

9.5.2 Primers

- Criteria for primers impacted by sequence variant will be:
    - Primer sequence has at least one mismatch to the genome in the last five base pairs from the primer's 3' end
    - Primer sequence has multiple mismatches to the genome with at least one mismatch landing in the 3' half of the primer
    - Primer sequence has no match to the genome

9.5.3 Probes

- Criteria for probe sequences predicted to be impacted are as follows:
    - Probe sequence has greater than two mismatches to the genome
    - Probe sequence has no match to the genome

9.5.4 For all criteria regarding impact, any mismatches caused by Ns or other ambiguous nucleotide nomenclature are ignored.

9.5.5 Risk for assay failure due to mismatch of primer or probe sequences will be assessed.

9.6 Analytical Specificity (Cross-Reactivity)

9.6.1 *In silico* analysis

- SARS-CoV2-2 specific primers and probes used in this assay will be compared to the sequences of pathogens in Table 6a using BLASTn with default settings:
  - The match and mismatch scores are 1 and -3, respectively
  - The penalty to create and extend a gap in an alignment is 5 and 2 respectively
  - parameters automatically adjust for short input sequences and the expect threshold is 1000.
- Homology will be evaluated based on % homology and orientation of primers and probes.

Table 6a. Summary of % Homology of Primer and Probes

Pathogen	Strain	GenBank Acc#	% Homology					
			N			*ORF1ab*		
			For Primer	Rev Primer	Probe	For Primer	Rev Primer	Probe
Staphylococcus epidermidis	ASM609437v1	NZ_CP035288.1						
Human coronavirus 229E	229E	NC_002645. 1						
Human coronavirus OC43	ATCC VR-759	NC_006213. 1						
Human coronavirus HKU1	HCoV-HKU1	NC_006577. 2						
Human coronavirus NL63	NL63	NC_005831. 2						
SARS-coronavirus	NA (isolate "Tor2")	NC_004718. 3						
MERS-coronavirus	NL140455	MG987421. 1						
Adenovirus (e.g. C1 Ad. 71)	type 2	J01917.1						
Human Metapneumovirus (hMPV)	CAN97-83	NC_039199. 1						
Parainfluenza virus 1 (Human respirovirus 1)	HPIV1/Los_ Angeles/USA/C H LA36/2016	MK167043.1						
Parainfluenza virus 2 (Human rubulavirus 2)	HPIV2/Seattle/US A/SC994 9/2018	MN369034. 1						
Parainfluenza virus 3 (Human respirovirus 3)	NIV1721711	MH330335. 1						
Parainfluenza virus 4a (Human rubulavirus 4a)	4a M-25	NC_021928. 1						
Influenza A	New York/392 /2004(H3N2)	NC_007373. 1, NC_007372. 1, NC_007371. 1, NC_007366. 1, NC_007369. 1, NC_007368. 1, NC_007367. 1,						

		NC_007370. 1						
Influenza B	B/Lee/1940	NC_002205. 1, NC_002206. 1, NC_002207. 1, NC_002208. 1, NC_002209. 1, NC_002210. 1, NC_002211. 1, NC_002204. 1						
Enterovirus (e.g. EV68)	coxsackievirus B1	NC_001472.1						
Respiratory syncytial virus	V13-0285	NC_030454. 1						
Rhinovirus	ATCC VR-1559	NC_038311. 1						
Chlamydia pneumonia	CWL029	NC_000922.1						
Haemophilus influenzae	Rd KW20	NC_000907. 1						
Legionella pneumophila	Philadelphia 1	NC_002942. 5						
Mycobacterium tuberculosis	H37Rv	NC_000962. 3						
Streptococcus pneumoniae	R6	NC_003098. 1						
Streptococcus pyogenes	M1 GAS	NC_002737.2						
Bordetella pertussis	Tohama I	NC_002929.2						
Mycoplasma pneumoniae	M129	NC_000912.1						
Pneumocystis jirovecii	RU7	NW_01726 4775.1						
Candida albicans	SC5314	NC_032089.1						
Pseudomonas aeruginosa	PAO1	NC_002516.2						

9.6.2 Wet testing evaluation will be performed with the cross-reactivity evaluation panel listed in Table 6b for samples collected in MTM media

9.6.2.1 Samples required: Specimens will be obtained from the American Type Culture Collection (ATCC) or BEI Resources and spiked into pooled negative samples collected in MTM

9.6.2.2 Study Design

- The SARS-CoV-2 assay will be performed on spiked samples in triplicate
- PCR will be performed with and without primers for *RPP30*

9.6.2.3 Interpretation

- Cross-reactivity will be defined as amplification by the SARS-CoV-2 assay with a Ct value $\leq$ 42.

9.6.3 Wet testing evaluation will be performed with the cross-reactivity evaluation panel listed in Table 6b for dry swabs

9.6.3.1 Study design (Dry swabs)

- Specimens will be obtained from the American Type Culture Collection (ATCC) or BEI Resources and spiked into pooled negative samples collected in MTM and applied to dry swabs

9.6.3.2 Study Design

- The SARS-CoV2 assay will be performed on spiked samples in triplicate
- PCR will be performed with and without primers for RPP30.

9.6.3.3 Interpretation

- Cross-reactivity will be defined as amplification by the SARS-CoV-2 assay with a Ct value $\leq$ 42.

Table 6b

Name/Description	**Concentration**
Human coronavirus 229E	1.6 × 106 TCID50 per mL
Human coronavirus OC43	104 ng/100 µL = 1.04 ng/µL
Human coronavirus HKU1	5.4 x 105 genome copies/µL 103 µL/vial
Human coronavirus NL63	1.6 × 105 TCID50 per mL
SARS-coronavirus	NA
MERS-coronavirus	8.9 × 105 TCID50 per mL
Adenovirus (e.g. C1 Ad. 71)	TCID50: 2.5 × 107 per mL
Human Metapneumovirus (hMPV)	25.8 ng per 100 µL
Human parainfluenza virus 1	0.41 ng/µL 102 ul in TE
Human parainfluenza virus 2	TCID50: 1 1.0 X 108 per mL
Human parainfluenza virus 3	0.201 ng/µL 10 ul in TE
Parainfluenza virus 4b	5.0 × 106 TCID50 per mL
Enterovirus (e.g. EV68)	117 ng per 100 µL
Rhinovirus	TCID50: 1 × 106 per mL
Haemophilus influenzae	8.4 x 105 genome copies/µL

Legionella pneumophila	6 µg/vial
Mycobacterium tuberculosis	1.6 mg/mL
Streptococcus pneumoniae	10 µg/vial
Streptococcus pyogenes	6 µg/vial
Bordetella pertussis	6 µg/vial
Mycoplasma pneumoniae	3.1 ng/µ 217 ng/vial
Pneumocystis jirovecii (PJP)	NA
Candida albicans	5.5 µg in 79 µL per vial (70 µg/mL)
Pseudomonas aeruginosa	7 µg/vial
Staphylococcus epidermis	6 µg/vial
Streptococcus salivarius	0.9 µg in 35 µL per vial (26 µg/mL)
Herpes Simplex virus	500000 c/ml
Varicella-zoster virus	100000 c/ml
Epstein Barr virus	94 µg/mL 11 µg/vial
Measles Virus	111 ng per 100 µL
Mumps virus	Conditions: Rhesus monkey kidney TCID50:11 X 105 – 1.6 X 106 per mL Conditions: Human amnion TCID50: 5 X 104 per mL Conditions: 8-day chicken embryo (allantoic) TCID50: 3.2 X 107 per mL
CMV	5E6 IU/mL
Corynebacterium diphtheriae	5 µg/vial
E.coli	5.9 µg in 26 µL per vial (227 µg/mL)
Lactobacillus plantarum	1.58 x 109 cfu/vial
Moraxella catarrhalis	9 µg/vial
Staphylococcus aureus	5.8 µg in 52 µL per vial (112 µg/mL)
Neisseria elongata	>104 cfu/vial
Neisseria meningitidis	1.0 µg in 31 µL per vial (31 µg/mL)
Chlamydophila pneumoniae	9.1×10^7 IFU/mL

9.7 Analytical Specificity (Interfering substances)

9.7.1 Samples required.

- Interfering substances to be tested are listed in Table 7a at the concentrations indicated.
- Pooled negatives collected in MTM will be spiked with SeraCare to serve as the matrix to test for inhibition
- Pooled negatives will be used as matrix to test for interfering signals

9.7.2 Study Design

- Each substance will be added to the pooled negative and contrived positive samples at the indicated concentration
- Samples will be tested in triplicate.
- PCR will be performed with and without primers for *RPP30*.

9.7.3 Interpretation

- Positive and negative controls must be valid
- A substance will be deemed an interfering substance if any positive sample is not detected.
- A substance will be deemed an interfering substance if any negative sample is detected.

9.7.4 Acceptance Criteria

- Samples with IC drop-out in a negative sample will be invalid and retested.
- 3/3 replicates must be valid to interpret results

Table 7a:

Substances	Stock Concentration	Evaluation Concentration
Afrin – nasal spray	100	15
Human blood	20	0.1
Human blood	20	0.5
Human blood	20	1
Chloraseptic	700	3.5
Flonase	100	5
Halls Relief Cherry Flavor	800	8
Nasocort Allergy 24 hour	100	5
Neo-Synephrine	100	5
Saline nasal spray	100	15
Zicam Cold Remedy	100	5

Purified mucin protein	2.5	0.06
Mouthwash	100	5
Rhinocort	100	1
Oseltamivir	175	2.5
Tobramycin	400	4
Zanamivir	400	3.3
Mupirocin	103	10
Peramivir	10,000	45

9.8 Sample stability

9.8.1.1 Previously tested clinical samples will be used for these experiments.

9.8.1.2 A negative control (0.3 mL of nCoV negative control from 2019-nCoV-PCR-AUS Kit) and a positive control (0.3 mL of nCoV positive control from 2019-nCoV-PCR-AUS Kit containing approximately 1000 copies), will accompany each sample plate.

9.8.1.3 Samples will be heat-inactivated and centrifuged before RNA extraction preparation.

9.8.1.4 Samples will be prepared for extraction by adding the following to a deep-well plate: 34 μL master mix consisting of 10uL Proteinase K, 4uL poly-A RNA and 20uL Internal Control per sample, 300μL of pooled samples, 300 μL of Lysis Buffer.

9.8.1.5 RNA from all samples will be extracted using the chemagic™ 360 running the "chemagic viral300 360 H96 prefilling VD200617.che" program and using the chemagic™ Viral 300 RNA/DNA Kit H96 kit.

9.8.1.6 RT-PCR Setup

9.8.1.6.1 SARS-CoV-2 RT-qPCR reagent Kit: 10 μL of each eluate will be mixed with 5 μL of a real time PCR master mix prepared using tubes nCoV Reagent A, nCoV Reagent B, and nCoV Enzyme Mix.

9.8.1.6.2 The reactions will be subjected to RT-PCR in an Analytik Jena 384.

9.8.1.7 Analysis

9.8.1.7.1 Data will be analyzed according to the Data Analysis SOP.

9.8.1.8 The laboratory has previously validated the PerkinElmer® New Coronavirus Nucleic Acid Detection kit. This study is to validate the sample stability of samples collected in MTM using the PerkinElmer® New Coronavirus Nucleic Acid Detection kit. Nasopharyngeal samples collected in MTM and previously extracted RNA will be used in the validation of this study.

9.8.1.9 Verification of Sample Stability

Sample stability testing will provide evidence of how the quality of the RNA varies with time under the influence of a variety of environmental factors such as temperature and humidity.

Sample RNA stability will be evaluated using a total of 80 previously tested and resulted samples. Of the 80 samples, 20 will not be detected in FAM or ROX, 20 will have a Ct range of 10-19 in either FAM or ROX, 20 will have a Ct range between 19-37, and 20 will have a Ct range between 37-42. All samples tested will have a passing IC (HEX_3) signal. Each of these samples will be tested at different times intervals: baseline, 24 hours, 48 hours, 96 hours, and 168 hours. In between each time interval, the samples will be placed back into a -80°C fridge for storage.

Sample stability for samples stored in MTM will be evaluated using the 80 previously tested and resulted samples from the RNA stability study. These samples will be tested at 3 different time intervals: baseline, 24 hours, and 72 hours. The samples will be stored at -80 in between the time intervals.

9.8.1.10 Compare CT values and diagnostic categories (not detected, detected, presumptive positive) between original run and repeat runs.

9.8.1.11 A sample will be considered invalid if the internal control fails (as per instructions for use by each RT-PCR assay) or instrument error.

9.8.1.12 All clinical samples should be positive for the internal control, otherwise marked invalid and not included in the analysis.

Acceptable Criteria Table	
Study	Acceptable Criteria
Verification of Sample Stability (RNA Stability Study)	20/20 (10%) Negative Samples will not be detected. 20/20 (100%) Samples Ct range 10-19 will be detected in FAM or ROX 18/20 (90%) Samples Ct range 19-37 will be detected in FAM or ROX 10/20 (50%) Samples Ct range 37-42 will be

	detected in FAM or ROX
Verification of Sample Stability (Sample Storage in MTM)	20/20 (100%) Negative samples not detected 20/20 (100%) Samples Ct range 10-19 will be detected in FAM or ROX 19/20 (95%) Samples Ct range 19-37 will be detected in FAM or ROX 16/20 (80%) Samples Ct range 37-42 will be detected in FAM or ROX

10. REFERENCES

10.1 Instructions for PerkinElmer® New Coronavirus Nucleic Acid Detection Kit v8.0

10.2 https://www.cdc.gov/coronavirus/2019-ncov/cdcresponse/about-COVID-19.html

10.3 https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html

10.4 CAP Molecular Pathology Checklist current edition

10.5 CAP Immunology Checklist current edition

10.6 CAP Laboratory General Checklist current edition

10.7 CAP All Common Checklist current edition

10.8 42 CFR Part 493 Laboratory Requirements

11. DOCUMENT HISTORY

Version	Summary of Changes	Date
1	New Document.	

Appendix A: Creation of Contrived Samples

A stock solution of LODs to be created using the following formula:

$$C1V1 = C2V2$$

Where:

C1 = Stock Concentration (5,000 copies/mL)

V1 = variable to be calculated

C2 = Desired Concentration (for example, 500 copies/mL)

V2 = 0.3 mL (volume of sample needed for extraction)

Example: For 500 copies/mL: 5,000x = 500 x 0.3 mL = 0.030 mL (or 30 μL)

- 6 μL will be added to 294 μL of Transport Medium to achieve 0.3 mL at 20 copies/mL
- The stock concentration (C1) for the SeraCare product must be obtained on a lot-by-lot basis from the SeraCare website.