SARS-CoV-2 PCR Assay Validation – Nasopharyngeal vs Saliva Sample Collection

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral load, as determined by polymerase chain reaction (PCR) threshold cycle (Ct) or quantitative values (genome copies/µL), generally peaks ~1-5 days after symptom onset [1-5]. Testing for the virus remains a key mitigation strategy preventing the spread of COVID-19. While nasopharyngeal swab PCR is considered the gold standard for SARS-CoV-2 detection, saliva sampling offers a simpler, less intrusive, cost-effective collection method [6]. Therefore, Celerion quickly and efficiently established and validated ultrasensitive SARS-CoV-2 assays for nasopharyngeal and saliva sampling as part of our internal mitigation strategy early during the pandemic. These methods were also applied to assess viral kinetics for during an 8-week observational study [7].

MATERIALS & METHODS

Nasopharyngeal and Saliva Quantitative PCR Assay

We established and validated an extraction-free ultrasensitive quantitative real-time polymerase chain reaction (qPCR) assay following CDC guidelines and primers/probe [8]. Nasopharyngeal samples were collected in viral transport medium (VTM, Hardy Diagnostics, Santa Maria, CA) and plated onto a 96-well optical plate (Applied Biosystems, Waltham, MA). Samples were heat inactivated and lysed for 5 min at 98°C in a thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems). Primer/probe sets for 2019-nCoV selected from the nucleocapsid gene (N1) and human RNase P gene (RP) (Integrated DNA Technologies, Coralville, IA), along with reaction mix (Reliance One-Step Multiplex RT-qPCR Supermix, BioRad, Hercules, CA) were added to the plate and loaded into the detector (7500 Real-Time PCR System, Applied Biosystems) for transcription and amplification. A standard curve was prepared using Heat-Inactivated 2019-nCoV (ATCC, Manassas, VA) and samples were reported as genome copies/µL.

Approximately 2mL of saliva was collected in a Saliva RNA Collection and Preservation System (Somru BioScience, Canada). Total nucleic acids were isolated with Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI). Transcription and amplification methods were performed as described above. The Heat-Inactivated 2019-nCoV standard curve and control samples were treated under the same conditions as the saliva samples, and results are reported as genome copies/µL.

Nasopharyngeal and Saliva qPCR Assay Validation

Both nasopharyngeal swab and saliva qPCR assays underwent a similar validation process unless otherwise stated. Control samples included: no template control (NTC), 2019-nCoV_N Positive Control (1:1000, 200 copies/µL, Integrated DNA Technologies), Hs_RPP30 Positive Control (1:1000, 200 copies/µL, Integrated DNA Technologies), pooled human buccal swab sample in VTM served as a human specimen control (HSC), and heat-inactivated virus in VTM was a quality control (100 copies/µL).

To evaluate assay precision and reproducibility, 3 runs were performed over a minimum of 2 days by minimum of 2 operators. Each standard/control were run in duplicate to determine within-assay precision. For the nasopharyngeal assay, the limit of detection (LoD) was determined by spiking Heat-Inactivated 2019-nCoV into VTM for 20 replicates. The samples were run in 3 dilutions near the estimated LoD in RT-PCR. For the saliva assay, Heat-Inactivated 2019-nCoV was spiked into saliva samples and RNA preservation media for 20 replicates. The samples were run in 1 dilution near the estimated LoD in RT-PCR. In both cases, the LoD was determined as the lowest concentration where ≥ 95% (19/20) of the replicates are positive for N1.

Clinical evaluation testing was performed by testing 30 positive and 30 negative samples in a randomized blinded fashion. The 60 samples were split into 2 groups. One group was spiked by Heat-Inactivated 2019-nCoV and the second aliquot was used as negative specimens. Among spiked (n=30), samples were contrived at 1X LoD (n=10), samples were contrived at 2X LoD (n=10), and the remainder were spiked spanning the assay testing range.

Short-term stability of N1 gene in nasopharyngeal or saliva samples were evaluated by processing 3 replicates of 2019-nCoV_N PC (1:1000) and Hs_RPP30 PC (1:1000) samples, which were aliquoted into a plate containing Bio-Rad Reliance One-Step Multiplex RT-qPCR Supermix maintained at 5°C for a minimum of 48 hours after addition into N1 and RP RT-PCR reaction mixes. Freeze-thaw stability (FT) of nasopharyngeal samples in VTM and saliva with preservation media were evaluated by processing 3 subjects in triplicate of spiked samples which had been stored at -80°C following a minimum of 4 freeze-thaw cycles. Sample stability was assessed against the same fresh samples spiked with Heat-Inactivated 2019-nCoV at 100 copies/µL and analyzed the same day of collection. The initial freeze cycle was for a minimum of 24 hours and all subsequent freeze cycles were for a minimum of 12 hours.

Nasopharyngeal Assay Performance

An aliquot of nasopharyngeal VTM obtained from our observational study [7], was sent to Quest Diagnostic Laboratories (Secaucus, NJ) to compare our internal assay results against a commercial emergency use authorized diagnostic test. Assay clinical performance was evaluated by contingency analysis and area-under the receiver operator curve (AUROC). Statistical analysis was performed with Prism, GraphPad (San Diego, CA).

RESULTS

Nasopharyngeal and Saliva qPCR Assay Validation

A total nucleic acid extraction-free method was first considered for both specimens, yet only the nasopharyngeal assay was successful. Therefore, here we describe the extraction-free nasopharyngeal and saliva (with nucleic acid extraction) qPCR validation requirements. Briefly, percent correctness was determined for within-assay, between-assay and between-operator precision and reproducibility, and was defined as agreement between runs. Overall, we observed 100% correctness for nasopharyngeal assay controls and standard curve concentrations ranging from 10000 to 0.5 genome copies/µL (Table 1).

Table 1. Within-Assay, Between-Assay and Between-Operator Precision for Controls and Standards

	Nasopharyngeal N1 and RP			Saliva N1 and RP		
%Correctness	**Within-Assay**	**Between-Assay**	**Between-Operator**	**Within-Assay**	**Between-Assay**	**Between-Operator**
NTC control	100%	100%	100%	100%	100%	100%
2019-nCOV_N PC (1:1000)	100%	100%	100%	100%	100%	100%
Hs_RPP30 PC	100%	100%	100%	100%	100%	100%
Quality Control	100%	100%	100%	100%	100%	100%
HSC control	100%	100%	100%	100%	100%	100%
Standard curve *	100%	100%	100%	100%** except 50% for 0.5 copies/µL	98.7%	98.7%

The following acceptance criteria was applied: NTC reactions for all primer and probe sets (N1and RP) do not exhibit fluorescence amplification curves that cross the threshold line. All reactions for 2019-nCoV_N PC and Hs_ RPP30 PC yielded a positive result, < 40.0000 Ct values, with the following primer and probe sets: N1 and RP respectively. All reactions for HSC yield a positive result, < 40.0000 Ct values, with the following primer and probe sets: RP; and do not exhibit fluorescence amplification curves that cross the threshold line for the following primer and probe: N1. *N1 was only measured for Standard curve with concentrations 10000-0.5 genome copies/µL. **All standard curve concentrations test achieved 100% within-assay correctness except for 0.5 genome copies/µL. NTC= no template control, PC= positive control.

While the saliva assay passed all validation test down to 1.0 genome copies/µL, in one run the 0.5 genome copies/µL standard curve concentration was undetermined. This suggests that the LoD for the saliva assay differed from the nasopharyngeal assay. LoD was defined as the lowest detectable copy number concentration of 2019-nCoV (N1) at which 95% of all (true positive) replicates tested positive. Indeed, in two separate runs of 0.5 genome copies/µL spiked samples fell below this threshold (Table 2), confirming the limit of detection at 1 genome copies/µL for the saliva assay compared to 0.5 genome copies/µL for the nasopharyngeal assay.

Table 2. Limits of Detection

	Nasopharyngeal Assay (Extraction-Free)			Saliva Assay (Extraction)		
LoD Analysis	**0.5 copies/µL**	**1.0 copies/µL**	**3.0 copies/µL**	**0.5 copies/µL**	**0.5 copies/µL**	**1.0 copies/µL**
N1 Mean Ct*	37.1695	36.0507	34.2025	36.3744	36.1418	34.709
N1 SD Ct*	0.7548	0.6130	0.2955	0.8649	0.7627	0.6425
N1 Positive/Total	20/20	20/20	20/20	16/20	18/20	20/20
% N1 Positive	100%	100%	100%	80%	90%	100%
% N1 Negative	0%	0%	0%	20%	10%	0%

*Calculations only include positive results. Positive samples defined as < 40.0000 Ct values for N1 primer and probe set.

Next, clinical evaluation of both assays demonstrated no amplification in the absence of the heat inactivated virus and positive results at 1x and 2x LoD as well as spiked concentrations spanning the assay test range (Table 3).

Table 3. Clinical Evaluation

Concentration (copies/µL)	N1 Positive/Total	% N1 Positive	RP Positive/Total	% RP Positive
Nasopharyngeal Assay (Extraction-Free)				
0	0/30	0%	30/30	100%
0.5	10/10	100%	10/10	100%
1	10/10	100%	10/10	100%
50	4/4	100%	4/4	100%
200	3/3	100%	3/3	100%
500	3/3	100%	3/3	100%
Saliva Assay (Extraction)				
0	0/30	0%	30/30	100%
1	10/10	100%	10/10	100%
2	10/10	100%	10/10	100%
50	4/4	100%	4/4	100%
200	3/3	100%	3/3	100%
500	3/3	100%	3/3	100%

Positive samples defined as < 40.0000 Ct values for the following primer and probe sets: N1 and RP. Negative samples defined as amplification in RP, but no amplification in N1.

Both freshly collected nasopharyngeal and saliva samples as well as those stored at 5°C were deemed viable during short-term stability testing. In addition, nasopharyngeal swabs in VTM and saliva in preservation media demonstrated integrity up to 4 freeze-thaw cycles established at -80°C (Table 4).

Table 4. Validation Results Comparison

Validation Components	Nasopharyngeal Assay (Extraction-Free)	Saliva Assay (Extraction)
Sample collection stability	Samples collected in viral transport medium (VTM) Stored at 2-8°C for 72 hr Stored at -80°C for long term	Samples collected with preservation media Stored at 2-8°C for 72 hr Stored at -80°C for long term
Precision and Reproducibility	100% reproducibility within-assay, between-assay and between operators	100% reproducibility within-assay, between-assay and between operators
Limit of Detection	0.5 genome copies/µL	1.0 genome copies/µL
Clinical Evaluation	100% negative for 0 genome copy/µL	100% negative for 0 genome copy/µL
	100% positive for other values	100% positive for other values
Freeze Thaw (FT) Stability	4 FT cycles established at -80°C	4 FT cycles established at -80°C
Short Term Stability	48 hours for N1 and RP reaction mix at 5°C	48 hours for N1 and RP reaction mix at 5°C

As a final validation step, we compared our assay results against a commercial diagnostic test. Our nasopharyngeal assay performed exceptionally, demonstrating 90% sensitivity, 84% positive predictive value and 93% AUROC (Table 5). Overall, we established two robust SARS-CoV-2 assays with extremely low LoD and excellent clinical performance.

Table 5. Internal Nasopharyngeal Assay vs Commercial Diagnostic SARS-CoV-2 Assay

Contingency Table	Commercial Results	
Nasopharyngeal Results	**Detected**	**Not Detected**
Detected	19	3
Not Detected	2	5

Assay Performance	Nasopharyngeal Assay	
Parameter	**Value**	**95% CI**
Sensitivity	0.9048	0.7109 to 0.9831
Specificity	0.625	0.3057 to 0.8632
Positive Predictive Value	0.8636	0.6667 to 0.9525
Negative Predictive Value	0.7143	0.3589 to 0.9492
AUROC (p-value)	0.9345 (0.004)	0.8403 to 1.029

DISCUSSION

Testing and tracing is a critical mitigation step during a pandemic. We established a set of robust, ultrasensitive PCR assays to prevent the spread of COVID-19 within our clinics but also to apply these techniques in an observational study. Our assays were able to detect virus levels down to 0.5 and 1.0 copies/µL for the nasopharyngeal and saliva samples, respectively. Both methods demonstrated excellent within-assay, between-assay and between operators reproducibility. Moreover, our extraction-free nasopharyngeal method reduced time and reagents for each run, resulting in a performance comparable to commercial diagnostic assays.

During the development phase, we found that the saliva assay did not perform optimally without the nucleic acid extraction step, therefore this step remained for saliva sample analysis, making a direct comparison between the two assays difficult. While others have successfully achieved extraction-free saliva assay development [9], we assume the proprietary reagents in the saliva preservation media may have interfered with the amplification process, requiring a nucleic acid extraction for the PCR run. Nonetheless, despite differences in assay methodology and sensitivity, when SARS-CoV-2 was detected in saliva samples in our observational study closely tracked with nasopharyngeal samples [7].

A recent meta-analysis of 16 studies that compared saliva to oropharyngeal or nasopharyngeal results found relatively high pooled sensitivity (88%) and specificity (92%) [10]. In our observational study [7], we observed comparably high specificity (83%) yet much lower sensitivity (37%). This difference in assay performance may be attributed to assay optimization mainly extraction-free vs nucleic acid extraction steps.

CONCLUSIONS

Overall, we established ultrasensitive SARS-CoV-2 qPCR assays with low limits of detection. The nasopharyngeal assay, was able to detect 0.5 genome copies/µL and also demonstrated excellent clinical performance, with faster turn-around time owing to a nucleic acid extraction-free approach. For saliva specimens, an extraction-free method was not suitable, however with nucleic acid extraction the assay limit of detection was 1 genome copies/µL. These assays were developed as part of Celerion's COVID-19 mitigation strategy early on during the pandemic, to ensure the safety of participants, employees, and visitors to its research facilities. In addition, these assays were applied in an observational trial, which followed asymptomatic and mildly symptomatic COVID-19 positive participants for 8 weeks [7].

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