

## Original Research Article

# Optimization of SARS-CoV-2 laboratory testing in a rural healthcare facility in the United States

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## ABSTRACT

**Background:** The diagnostic testing for SARS-COV-2 (COVID-19) presented a profound challenge to the entire world, dominating the concern of most governments and public health systems, particularly rural community hospitals in the United States. Indiana University of Pennsylvania (IUP) in partnership with Indiana Regional Medical Center (IRMC) began on site, same-day COVID-19 testing in efforts to not only combat the challenges that health providers faced in rural Indiana community but also help to strengthen global diagnostic capacity.

**Methods:** Clinical samples were collected as dry swabs from the nasopharyngeal (NP) regions and processed in phosphate buffer saline (PBS). The crude RNA was directly tested using real-time (RT) reverse transcription quantitative polymerase chain reaction (RT-qPCR) with PrimeDirect probe RT-qPCR Mix (Takara Bio USA) and optimized with probe-primer sets [Integrated DNA Technologies (IDT)].

**Results:** Validation experiments with dry swabs from NP clinical samples showed no difference in the testing accuracy to those collected in viral transport medium or universal transport medium. Extraction of COVID-19 RNA in PBS reduced processing time of a batch of 50 NP clinical samples from 6 hours to an hour. This allowed for rapid diagnostic testing of nearly 200 clinical samples per day. Optimization of analytical variables helped to detect virus loads up to 2.0 copies/μl during routine diagnostic testing.

**Conclusions:** During an infectious outbreak, the ideal response by public health authorities is rapid testing. The collaboration between IUP and IRMC attests to the importance of teamwork between local initiatives to detect and prevent further spread within a rural community.

**Keywords:** COVID19, Community medicine, RT-PCR, Rural health

## INTRODUCTION

Several nations around the world are at a pivotal stage in the fight against the COVID-19 known to be caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). The SARS-CoV-2 belongs to the Coronaviridae family and has a positive viral RNA genome that codes for spike (S), membrane (M), envelope (E), and nucleocapsid (N) structural proteins. The virus is highly insidious and spreading faster killing

thousands of individuals worldwide and the disease has been classified as a pandemic.<sup>1</sup> Vaccines have been developed in record time and are finally available, yet challenges prevail. While vaccines are crucial components of the response strategy, they alone will not obviate the virus. The WHO has repeatedly emphasized the importance of continued testing to break the chains of transmission and prevent further infections.<sup>1,2</sup> Clinical evaluations of symptomatic patients with COVID-19 are generally nonspecific, that include fever or chills, cough,

fatigue, shortness of breath, new loss of taste or smell with some patients being completely asymptomatic.<sup>1</sup> In severe cases COVID-19 has been implicated in acute respiratory distress syndrome, resulting in respiratory failure and death. Therefore, precise diagnostic testing followed by short turnaround time (STAT) is central to identifying symptomatic patients, managing the disease, and establishing regular surveillance of asymptomatic carriers that warrant suitable isolation measures to prevent further disease spread.

Very early during the pandemic most diagnostic tests for COVID-19 in the United States included COVID-19 RT-PCR (LabCorp); 2019-novel coronavirus real-time RT-PCR Diagnostic Panel [U.S. Centers for Disease Control and Prevention (CDC)]; and TaqPath COVID-19 Combo kit (Thermo Fisher-Applied Biosystems).<sup>3-5</sup> Serology hitherto has had a significant role in the epidemiology of SARS and other coronavirus outbreaks.<sup>6,7</sup> Serological assays have operationally expanded for the rapid diagnosis of SARS-CoV-2 as an excellent supplemental tool for COVID-19 testing that have a huge potential for the epidemiology of COVID-19.<sup>8-10</sup> For a thorough review of the significance of these tests see commentary.<sup>8</sup> The U.S. Food and Drug Administration (FDA) has granted EUA's for serological assays that detect antibodies to two of the four structural proteins (see FDA's list of in vitro Diagnostics EUAs) that can identify SARS-CoV-2. It is clear from several published reports that serological methods may play a role in confirming late COVID-19 infections and have the advantage of STAT and low-cost detection of SARS-CoV-2.<sup>8,9</sup> However, the test results are greatly impacted by the limit of sensitivity and specificity of detection in early infections given the unpredictability of viral loads in COVID-19 patients (IRMC unpublished data). Similar assessments on serological tests have been made for other coronavirus strains.<sup>11,12</sup> Hence serological tests may not play a significant role in immediate patient management.<sup>10</sup> The "gold standard" for clinical diagnostic detection of SARS-CoV-2 is nucleic acid amplification test (NAAT) like real-time reverse transcriptase-polymerase chain quantitative assays (rRT-qPCR) and is highly recommended for molecular testing.<sup>13-15</sup> In literature there are several published reports of optimization of preanalytical processes including types of swab, specimen collection and the risks involved in aerosolization and transmission to health care workers.<sup>16</sup> SARS-CoV-2 diagnostic testing relies on accurate sampling techniques, region of sampling, and adequate sample recovery from stored transport media.<sup>11,12</sup> Sampling from the nasopharyngeal (NP) region is recommended for the detection of SARS-CoV-2 since it provides the highest viral load and rate compared to those from the oropharynx and nasal cavity.<sup>2,17-19</sup> One of the biggest challenges to diagnostic testing of COVID-19 has been the growing demand globally for several pre-analytical tools and test kits that are in short supply in various hospitals predominantly in community hospitals serving rural populations.<sup>20</sup>

The primary objective of this study was to eliminate testing limitations and promote in-house COVID-19 testing at IRMC. For this purpose we investigated several process variables including viral transport media (VTM), Universal Transport Media (UTM), RNA extraction in PBS from dry NP swabs, and assessed SARS-CoV-2 detection using PrimeDirect Probe rRT-qPCR reagent kit (Takara Bio) with optimized combination of CDC EUA authorized primer sets and probes for the two loci of the nucleocapsid protein markers (N1 and N2) with a confirmatory assay using RPP30 gene (SARS-CoV-2 (2019-nCoV) using CDC approved qPCR Probe Assay (Integrated DNA Technologies, USA).<sup>21</sup>

## METHODS

The study involved more than 10,000 patients and the patient criteria included individuals with symptoms associated with SARS-CoV2 infection, patients who meet CDC definition of close contact with infected persons, and individuals who are scheduled for procedures for which the hospital has required pre-procedure COVID testing. All testing was done at Indiana regional Medical Center (IRMC) in Indiana, Pennsylvania, USA. Approvals for testing, data collection, and publication have been approved by the IRMC IRB. All nasopharyngeal specimens were collected by trained healthcare workers at IRMC in accordance with CDC's protocols and guidelines.

### *Nasopharyngeal (NP) specimens*

The bridge experiments and validation studies for pre-analytical and analytical variables were done with over 100 NP clinical specimens. Individual swabs collected in 2-3 ml of VTM or UTM or in 0.25 ml of PBS were used in the study. These specimens originated from outbreaks of coronavirus diseases in Indiana, Pennsylvania between March, and December 2020.

### *Procedures*

#### *Extraction and purification of RNA from NP specimens*

##### *Spin column SARS-CoV-2 viral RNA extraction (BIO-BASIC) from samples stored in VTM and UTM*

In the early stages (March-early July 2020) of the COVID-19 outbreak, SARS-CoV-2 viral RNA was purified using BIOBASIC kit. The NP swab samples were transferred to a sterile microtube containing 0.2 ml of PBS and vortexed vigorously for 30 s. Each sample was then treated with 0.6 ml freshly prepared lysis master mix containing 10 µg of the carrier RNA (BIOBASIC). Samples were vortexed vigorously intermittently for 10 min and incubated with equal volumes of cold absolute ethanol by repeated inversion. All such samples (600 µl) were transferred to a spin column provided by the supplier and centrifuged at 10,000 g for 1 min. The flow through was discarded and the column was washed twice with 0.5 ml RPE solution (BIOBASIC) and viral-RNA was eluted with 60 µl of

RNase free water. The purified viral RNA was immediately used for rRT-qPCR assays using SARS-CoV-2 RT-PCR detection kit (BIOBASIC) or PrimeDirect™ Probe RT-qPCR Mix (Takara Bio) depending on the availability of the kits from the manufacturers.

#### *Direct SARS-CoV-2 Viral RNA Extraction from dry swabs*

With greater demand for diagnostics testing and screening at IRMC, the collaborative effort between IUP and IRMC helped to pilot Takara's PrimeDirect™ Probe RT-qPCR Mix for diagnostic testing. We have since applied for EUA addendum to our testing protocol. The NP samples were treated with 0.25 ml of PBS and vortexed vigorously for 30 seconds and 2.5 µl of the crude suspension was directly used for testing in rRT-qPCR assays using PrimeDirect™ Probe RT-qPCR Mix without further processing. The choice of primer-probe combination as per CDC's Emergency Use Authorization protocol <https://www.fda.gov/media/134922/download> for 2019-Novel Coronavirus. TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Black Hole Quencher 1 (BHQ-1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end.

#### *rRT-qPCR assays using BIO Basic detection kit*

During the months of April to June 2020, rRT-qPCR assays for COVID-19 testing were done using detection kit BIO BASIC. Each reaction included 1 µl of EZ-RT-PCR master mix, 19 µl of COVID-19 primer probe buffer mix and 5 µl of NP specimen sample. The RT-qPCR assays were performed on Eppendorf Mastercycler® ep realplex system, using the following parameters for a sample volume of 25 µl with accepted default values of ramp rate settings 4 °C/s. Each reaction mix was thoroughly mixed before adding to the well plates. The thermal-cycling conditions included cDNA synthesis at 55°C for 15 min followed by 1 cycle RT inactivation at 95°C for 30 seconds; and amplification of 40 cycles at 95°C and 60°C for 1 minute.

#### *RT-qPCR assays using PrimeDirect™ Probe RT-qPCR Mix*

During the pandemic outbreak in the days of July 5-July 20 we piloted RT-qPCR assays of SARS-CoV-2 RNA purified from VTM or UTM, or dry swab using PrimeDirect™ Probe RT-qPCR Mix (Takara Bio). The reaction set up and qPCR conditions for SARS-CoV-2 RNA detection were done as per manufacturer's instructions. A 25 µl PCR reaction set up included 12.5 µl PrimeDirect Probe RT-qPCR Mix (2X) along with PCR forward and reverse primers 0.5 µl each (10µM) and 0.5 µl probe (final concentration 0.2 µM) in primer buffer mix with 5 µl of NP specimen sample. The rRT-qPCR assays were performed on Eppendorf Mastercycler® ep Realplex system, using the following parameters with accepted default values of ramp rate settings 4°C/s. Each reaction

mix was thoroughly mixed before adding to the well plates. The thermal cycling conditions: reverse transcription 90°C for 3 min and 60 °C for 5 min followed by PCR reaction of 40 cycles at 95°C 5 seconds and 55°C for 30 seconds. For all validation and bridge experiments changes were made to the reagent input described above.

#### *Validation and Bridge Experiments*

##### *Standard curve and reproducibility of the assay using PrimeDirect™ Probe RT-qPCR Mix*

The standard curve was generated using 10-fold serial dilutions of the control plasmids that contain the complete nucleocapsid gene from 2019-nCoV (IDT catalog # 1000625). The 2019-nCoV CDC qPCR Probe Assays target regions within the nCoV nucleocapsid gene N1 and N2 which is present in the 2019-nCoV\_N Positive Control plasmid. For the Hs\_RPP30 positive control a portion of the RPP30 gene (IDT Catalog # 1000626) was used that harbor a single copy gene in the human genome. The plasmid dilution ranged from 10<sup>-1</sup> to 10<sup>-5</sup> representing 2.0 × 10<sup>4</sup> to 2.0 copies/µl and was tested in triplicate as described above. The standard curve was plotted between the standard plasmid concentration (log copy number) and cycle threshold (Ct). In addition, the standard curve was generated from 10-fold serial dilutions of NP specimen samples positive for SARS-CoV-2 RNA prepared from dry swabs of infected individuals. To confirm the reproducibility of the RT-qPCR protocol, both intra-assay and inter-assay were evaluated using 10-fold serial dilutions of plasmid containing N1, N2, and RP genes ranging from 10<sup>-1</sup> to 10<sup>-5</sup>. As a measure of variance within an assay both the assays were performed in triplicate in a single run or three independent times. The measured Ct values obtained from each assay were calculated to determine the standard deviation (SD) and coefficient of variation (%CV), which were used to assess the reliability of the RT-qPCR protocol. All plasmid samples, including the no template control were run in triplicate and each qPCR reaction was repeated twice.

##### *Optimization of SARS-CoV-2 RNA detection of dry swab NP specimens using PrimeDirect Probe RT-qPCR Mix*

Various reaction volumes of the master mix along with primer probe concentrations were assessed both with positive controls and NP positive specimen samples. In the optimization experiments 10 positive and negative NP specimens and control plasmids that contained complete nucleocapsid gene (N1, N2, and RPP30) purchased from IDT were used in replication of three to validate. In all such assays reaction volumes ranged between 5 µl and 20 µl with 2.5 µl of the NP specimens prepared from dry swabs described above. As per manufacturer's instructions (Takara Bio), the amount of PrimeDirect Probe RT-qPCR Master mix used was always less than 10% of the PCR reaction mixture volume to get optimum results. A 17.5 µl PCR reaction set up included 2.0 µl PrimeDirect Probe RT-qPCR Mix (2X) along with PCR forward and reverse

primers 0.25 µl each (10µM) and 0.25 µl probe (final concentration 0.2 µM) in primer buffer mix with 2.5 µl of NP specimen sample. The rRT-qPCR assays were performed on Eppendorf Mastercycler® ep Realplex system, using the following parameters with accepted default values of ramp rate settings 4°C/s. Each reaction mix was thoroughly mixed before adding to the well plates. The thermal cycling conditions: reverse transcription 90°C for 3 minutes and 60°C for 5 min followed by PCR reaction of 40 cycles at 40 for 95°C 5 seconds and 55°C for 30 seconds. For all validation and bridge experiments changes were made to the reagent input described above. All NP samples and positive control plasmids, including the no template control and human specimen samples negative for COVID-19, were run in triplicate and each qPCR reaction was repeated twice.

#### *Detection limit of SARS-CoV-2 RNA in clinical samples using PrimeDirect Probe RT-qPCR Mix*

To compare the sensitivity of SARS-CoV-2 RNA isolation from dry swabs and detection in clinical samples, RT-qPCR assays were performed from 10 NP specimens positive for COVID-19. SARS-CoV-2 RNA was isolated from dry swabs as previously described. The RNA sample was 10-fold serially diluted and processed for rRT-qPCR analysis. The 10-fold serial dilutions of the sample RNA suspension were prepared as described, and 2.5 µl of each dilution was used for rRT-qPCR assays for a final volume of 17.5 µl reaction. Each dilution was performed in triplicate for each of the sample for all the three genes. All positive control plasmids, including the no template control and human specimen samples negative for COVID-19, were run in triplicate and each qPCR reaction was repeated twice.

Statistical analysis and calculations were completed using Microsoft Excel. Standard curves between mean Ct values and log copy number were fit using the LINEST function in MS Excel assuming a linear relationship.

Raw fluorescence readings were exported from Opticon Monitor software and processed in MS Excel. The procedure for data processing in real-time PCR procedure included: i) Noise filtering from raw fluorescence readings by smoothing, baseline subtraction and amplitude normalization; ii) The optimal threshold was selected automatically from regression parameters of the standard curve; iii) The means and their variances were calculated for all data points in triplicates for N1, N2, and RP genes. The Comparisons between mean Ct values from UTM, VTM, and dry swabs were made by qualitatively comparing the values of the means and standard deviations.

The coefficient of variation was calculated as a measure of reliability among intraassay replicates.

## RESULTS

### *Comparison of SARS-CoV-2 RNA extraction, PCR components and detection methods*

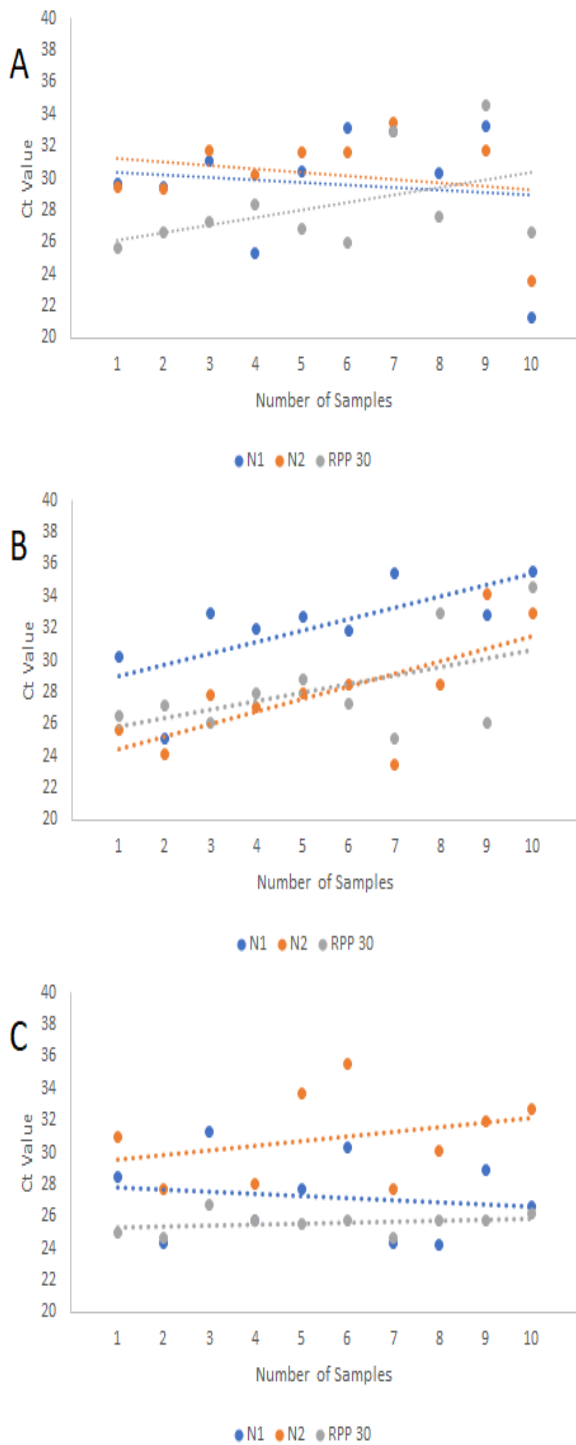
In our effort to expedite COVID-19 testing, several published and EUA approved protocols were used for SARS-CoV-2 RNA extraction and detection. At maximum, 100 NP specimens collected in VTM or UTM could be tested per day BIOBASIC. The limitation of the availability of the resources like transport media, RNA purification kit, and the number of samples that could be tested was overcome by collecting dry swabs adding PBS and directly testing with PrimeDirect Probe RT-qPCR Master Mix. In all, a maximum of 200 NP patient samples/day could be tested.

### *Validation and reproducibility of rRT-qPCR assays using dry swab NP specimens*

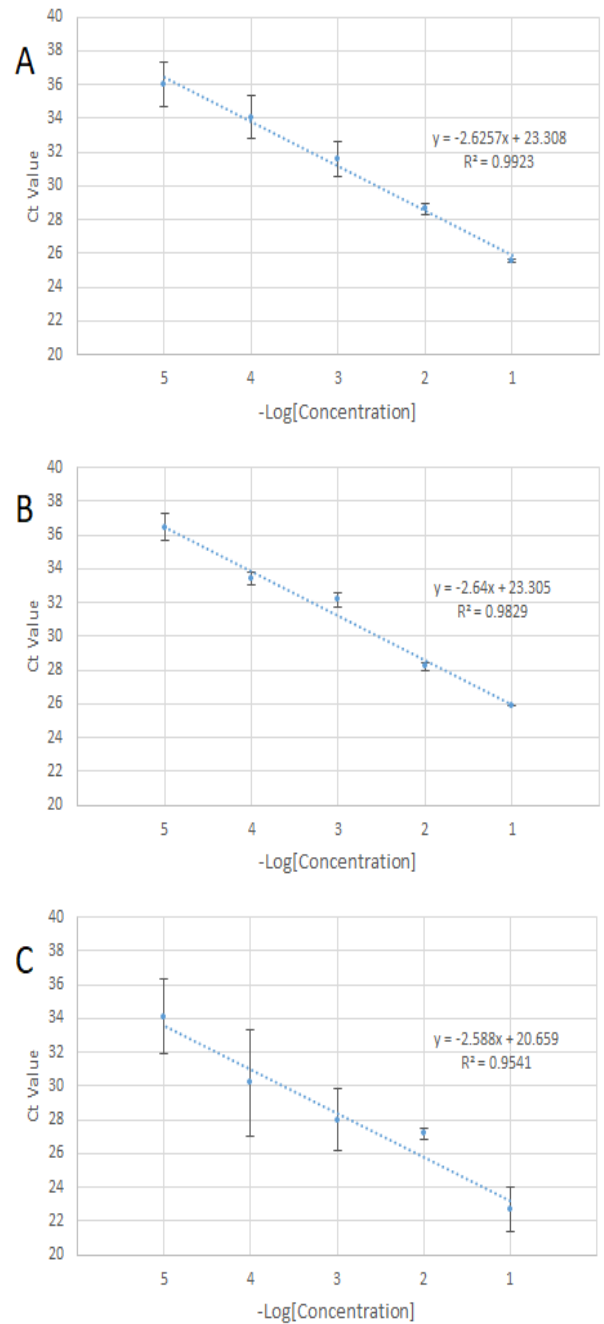
During the early onset of the pandemic between February and May 2020, rRT-qPCR assays were performed routinely on VTM and UTM NP patient samples in accordance to CDC guidelines. Due to greater demand of these pre-analytical variables and extreme shortage in rural community hospital, an effort was made to validate other sampling methods to accommodate testing for the critical group in rural community hospitals. To overcome these limitations, a batch of NP swabs collected as dry swabs from 10 patient samples were compared with that many subject specimens in VTM and UTM. All SARS-CoV-2 RNA for RT-qPCR validation in this experiment were tested by PrimeDirect Probe RT-qPCR Master mix and EUA primer probe kit supplied by IDT. As per FDA recommendations, SARS-CoV-2 RNA was analyzed for N1, N2, and RP genes (Figure 1). The mean Ct values of N1 were 29.7, 28.8 and 27.1 and N2 was 27.2, 27.9, 27.8 and RPP30 were 28.2, 28.2, and 25.5, respectively, in VTM, UTM, or dry swabs.

### *Standardization of RT-qPCR assay and reproducibility using PrimeDirect™ Probe RT-qPCR Mix*

As per the FDA recommendations, the standard curve of the RT-qPCR assay was determined using 10-fold serial dilutions  $10^{-1}$ - $10^{-5}$  dilutions which is equivalent to  $2.0 \times 10^4$  to 2.0 copies/µl of the control plasmids containing the complete nucleocapsid gene for 2019-nCoV (Figure 2). The mean Ct values of each dilution for each of the three genes tested N1, N2, and RPP30 were reproducibly obtained from three replicates (Figure 2). dilutions from 3 NP specimen samples collected from COVID-19 infected patients as dry swabs produced a standard curve with the slope and amplification efficiency close to plasmid DNA positive controls (data not shown). In addition, during validation, tests of 3 NP samples collected from infected patients at various concentrations yielded consistent results over a range of five dilutions for N1, N2, and RPP30 genes.



**Figure 1: Comparison of Ct values of 30 select NP specimen: SARS-CoV-2 RNA in VTM, UTM, and dry swabs in PBS. NP specimens in VTM, UTM and those collected as dry swabs were tested for N1, N2, and RPP30 genes as per CDC guidelines. Clinical samples that were negative for each of the variables had no Ct values. All data points were in triplicate.**



**Figure 2: Standard curve of FAM based rRT-qPCR probe detection of positive control plasmid: Each positive control reaction also included control plasmids that contained complete nucleocapsid gene from 2019-nCoV\_N and Hs\_RPP30 positive controls. The Hs\_RPP30 Control contains a portion of the RPP30 gene, a single copy gene present in the human genome (IDT technologies). The standard curve was plotted between mean Ct values obtained from each dilution of standard plasmid against calculated log copy number (A) N1 slope=-2.6257,  $R^2=0.9923$ ; (B) Standard curve of N2 showed slopes=-2.64,  $R^2=0.9829$  and (C) Standard curve of RPP30 showed slopes=-2.588,  $R^2=0.9541$ .**

**Comparison and Optimization of SARS-CoV-2 RNA detection of dry swab NP specimens using PrimeDirect Probe RT-qPCR Mix**

As per FDA regulations, bridge experiments were done to optimize the use of analytical variables. The rRT-qPCR assay was tested for 5, 10, 15, and 20 µl reaction volumes and compared with manufacturers’ recommendations (25 µl). These results were comparable with the manufacturer’s protocol for 25 µl. One of the parameters in fluorescent detection using PrimeDirect Probe RT-qPCR Master Mix to consider was the oligonucleotide probes labeled with a 5' fluorophore (FAM) and a 3' quencher (BHQ1) with the PCR primers to be less than 10% of the PCR reaction volume.

**Reproducibility of rRT-qPCR assay**

The reproducibility of the assay was characterized by analysis of assay variations (Table 1). The assay was

analyzed by measuring the mean Ct values of independent runs over three days using 10-fold serial dilutions of standard control plasmids (10<sup>-1</sup>-10<sup>-5</sup>). The coefficient of variation (%CV) calculated from measured mean Ct values ranged from 0.43% to 3.57% with SD values from 0.11 to 1.28 for N1; 0.02% to 2.18% with SD values from 0.005 to 0.79 for N2; and 1.18% to 10.48% with SD values from 0.32 to 3.17 for RPP30. Moreover, the %CV of mean Ct values of the intra-assay was 0.41%-13.55% with SD values from 0.08-3.13 for N1; 0.44%-14.93% with SD values 0.46-3.83 for N2; and 0.15%-15.99% with SD values 0.04-4.8 for RPP30 of the three NP clinal samples tested Table 1. Plasmid controls for MERS, SARS and human specific RNA samples were included in each reaction as negative controls to further determine the specificity of rRT-qPCR primers and probes for any non-specific amplification.

None of these samples and the no template controls yielded any result.

**Table 1: Reproducibility of intra-assay and inter-assay with 10-fold serial dilution of standard positive control DNA and select NP specimen samples for N1 and N2 and RPP30 genes. All positive control plasmids and NP samples were serially diluted in PBS. The rRT-qPCR assays were done with 2.5µl template in 17.5µl reaction volume.**

Plasmid concentrations Copy no/µl	Positive control (N1)			Positive control (N2)			Positive control (RPP30)		
	Mean Ct	SD <sup>a</sup>	CV (%)	Mean Ct	SD <sup>a</sup>	CV (%)	Mean Ct	SD <sup>a</sup>	CV (%)
2.0×10 <sup>4</sup>	25.59	0.11	0.43	25.87	0.005	0.02	22.67	1.31	5.8
2.0×10 <sup>3</sup>	28.66	0.32	1.14	28.21	0.24	0.85	27.16	0.32	1.18
2.0×10 <sup>2</sup>	31.58	1.03	3.28	32.15	0.42	1.38	27.99	1.84	6.6
2.0×10 <sup>1</sup>	34.08	1.23	3.62	33.41	0.35	1.07	30.18	3.17	10.52
2.0×10 <sup>0</sup>	36.01	1.28	3.57	36.47	0.79	2.18	34.1	2.21	6.48
<b>SARS-CoV-2 RNA Dilution</b>	<b>(N1)</b>			<b>(N2)</b>			<b>(RPP30)</b>		
<b>NP Specimen 1</b>									
2.0×10 <sup>4</sup>	18.6	1.97	10.64	21.9	1.71	7.81	28.9	0.33	1.14
2.0×10 <sup>3</sup>	19.4	1.39	7.17	22.2	2.97	13.35	30.8	2.34	7.58
2.0×10 <sup>2</sup>	20.5	0.08	0.41	22.8	2.12	9.28	33.6	2.48	7.38
2.0×10 <sup>1</sup>	22.43	1.6	7.15	24.3	0.65	2.66	34.9	2.66	4.04
2.0×10 <sup>0</sup>	24.51	3.13	12.8	27.14	1.18	0.44	34.7	0.44	1.28
<b>NP Specimen 2</b>									
2.0×10 <sup>4</sup>	23.1	2.02	8.77	26.4	0.46	1.76	31.6	0.04	0.15
2.0×10 <sup>3</sup>	24.04	1.83	7.64	26.7	1.13	4.25	33.4	0.53	1.5
2.0×10 <sup>2</sup>	25.4	0.96	3.8	28	0.7	2.49	34.7	0.96	2.76
2.0×10 <sup>1</sup>	27.8	2.45	8.81	29.7	2.15	7.24			
2.0×10 <sup>0</sup>	29.4	2.68	9.11	30.6	2.79	9.11			
<b>NP Specimen 3</b>									
2.0×10 <sup>4</sup>	21.4	0.95	4.45	23.9	2.59	10.85	29.32	2	6.84
2.0×10 <sup>3</sup>	23.38	0.79	3.41	25.4	1.13	4.47	30.24	4.8	15.99
2.0×10 <sup>2</sup>	24.66	1.03	4.18	25.6	3.83	14.93	30.9		
2.0×10 <sup>1</sup>	24.82	3.04	12.25	26.8	1.3	4.87	32.7	2.73	8.36
2.0×10 <sup>0</sup>	27.07	3.66	13.55	28.81	2.43	8.4	33.5		

## DISCUSSION

Due to nature of the COVID-19 pandemic and the immense clinical and epidemiological consequence, several clinical laboratories in the US, including those associated in-house with the rural community hospital at IRMC, were required to perform validation and bridging studies on US FDA approved diagnostic testing to implement testing under the EUA regulation.

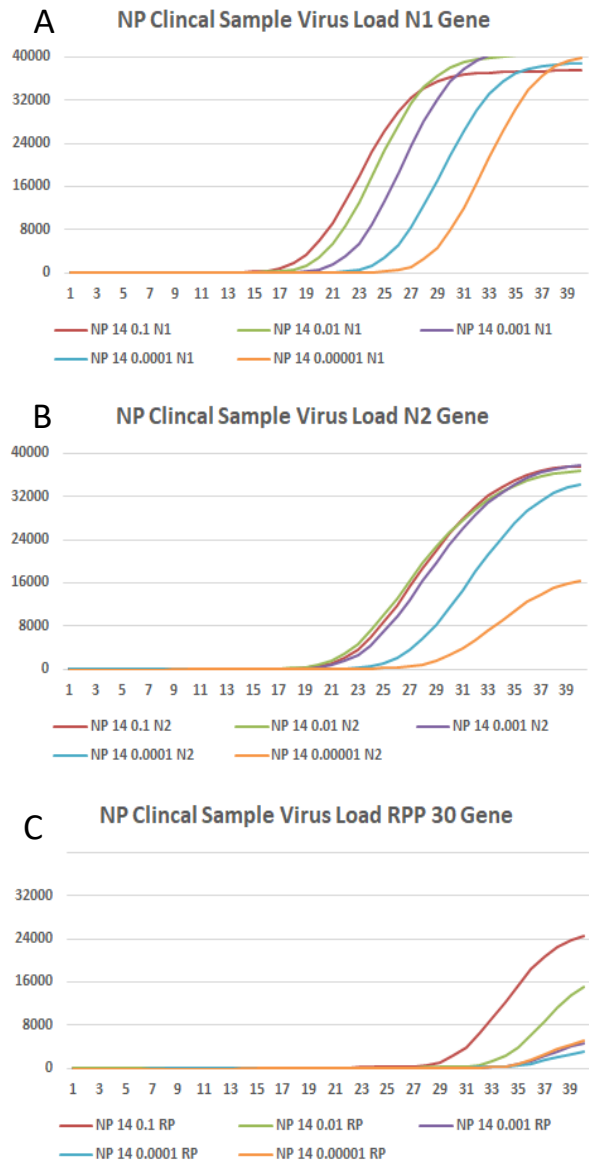
Through the IUP-IRMC partnership, we have advanced a Laboratory Developed Tests (LDT) that meets all guidelines outlined by Clinical Laboratory Improvement Amendment (CLIA) standards and College of American Pathologist (CAP). Additionally, during the first eight months of the epidemic in the United States most hospitals and communities were suffering from failure to get testing done in a clinically valid timely manner. It is known that when the virus is circulating in a community, the time from symptoms or exposure to getting a test result needs to be short. If there is a gap that is greater than 72 hours contact tracing is nearly impossible.

Other asymptomatic exposed people have too much time to spread the virus. From February-July there were many hospitals and clinics that had to wait 15 days to get results of COVID-PCR testing. IUP-IRMC RT-turnaround time is 12-24 hours. Moreover, there was a critical need to lower costs by minimizing input use since many of the published methodologies for COVID-19 testing, including RNA extraction, were time consuming, expensive, and more often reagents were in short supply. Hence, a 50 µl or 25 µl reaction volume was prohibitive in testing. To address these specific limitations during the initial stages of the pandemic, early validation experiments were performed on several variables to optimize testing in a rural setting hospital including i) on collection methods using dry swabs; ii) extraction of SARS-CoV-2 viral RNA; and iii) optimizing RT-qPCR assays using PrimeDirect probe with low volume rRT-qPCR conditions.

The initial validation and bridge experiments with NP patient samples showed no difference in the testing accuracy of SARS-CoV-2 viral RNA from NP swab samples without the use of VTM or UTM (Figure 1). The mean Ct values from rRT-PCR assays of 10 such clinical samples collected as dry swabs were comparable to those from VTM and UTM. These values were between 25.5 and 27.8 for N1, N2 and RPP30.

The CAP and CLIA guideline outlined protocol using dry swab NP specimens has immensely reduced SARS-CoV-2 viral RNA processing time of a batch of 50 NP test samples from 6 hours to an hour. Thus, allowing for rapid diagnostic testing of up to 200/day using PrimeDirect Probe RT-qPCR Master mix. Thus, the in-house optimization and testing using rRT-qPCR assays in alternative best option for a reliable test with good STAT for many decisions the physician make in caring for COVID-19 patients and other medical emergencies. In

addition, by directly using dry swabs with SARS-CoV-2 viral RNA on PBS, we have not only avoided several intermediate steps in RNA processing that influence the performance these tests but also reducing the risk of cross contamination and greatly diminishing the exposure time of laboratory personnel to the virus.



**Figure 3: Development of a multiplex rRT-qPCR assay for N1, N2, and RPP30 genes from a representative clinical sample assessing SARS-CoV-2 virus load. Individual forward, reverse, and probes for SARS-CoV-2 RNA N1, N2, and RP genes for were from IDT Technologies as part of the EUA kit. The final concentration in each reaction was 500 nM for the forward and reverse primers and 125 nM for each probe. In the above figure panels (A),(B) and (C) represent SARS-CoV-2 RNA assay results from a dry swab processed in PBS. The crude RNA was serially diluted on PBS and 2.5 µl of the RNA was used in 17.5 µl reaction volume.**

The data presented here shows broad comparisons of three different commercially available diagnostic kits with fivefold reduction of PCR reagents that produces reliable results. These results were accurate and reliable for quantification of dry swab NP patient samples and positive plasmids that contained complete nucleocapsid gene from 2019-nCoV\_N and Hs\_RPP30 controls. Specifically, the correlation coefficient (R2 value) of the standard curve from the positive control plasmids and COVID-19 infected NP specimens from dry swabs showed an efficiency of higher than 96% indicating the high precision of the assay. False-positive test results without optimization of primer-probe sets have been reported previously.<sup>22-25</sup> Optimization of the forward and reverse primers with a final concentration of 500nM for N1, N2, and RPP30 and 125 nM probe of in each PrimeDirect Probe RT-qPCR Master mix PCR mixture was critical for our target specific diagnostic testing of COVID-19.<sup>26</sup>

The Ct values of all COVID-19 infected patients ranged between 15.56 and 24.19 for N1; 17.4 and 31.35 for N2; and 23.82 and 28.35 for RPP30 genes with no amplification with negative controls. Comparison of reaction volumes of Prime Direct Probe master mixes and optimization studies suggest that SARS-CoV-2 RNA could be detected in reaction volumes as low as 5 µl (Figure 3).

To further improve our testing capacity and address the analytical inputs, several bridge experiments and validation tests gave consistent results and the assay was linear over 4 orders of magnitude in a reaction volume of 15µl with a 20% reduction in the PrimeDirect Probe RT-qPCR Master mix. Comprehensively, the assay can also differentiate between symptomatic and asymptomatic individuals suggesting that dry swab collection method could be applied for routine diagnosis (data not shown). Indeed, the viral load in individuals vary considerably, the dry swab collection and detection using PrimeDirect probe in the rRT-qPCR method demonstrated that the assay could detect SARS-CoV-2 viral RNA. Notably, we observed range of virus load from 2.0 to  $2 \times 10^{10}$  copies/µl in some patients during routine diagnostic testing like those reported.<sup>27</sup> Although, it is difficult to compare the levels of virus between the individual patient samples and laboratory derived control plasmids as we do not know when the symptomatic and asymptomatic acquire the infection.<sup>28</sup> Many factors such as the dates of sample collection, the level of virus in an individual, the immune response of each patient, as well as different days of infection, may contribute to the level of SARS-CoV-2 viral RNA in individual samples.<sup>27</sup>

The COVID-19 pandemic and diagnostic testing for SARS-COV-2 presented a deep challenge to the entire world, with some areas impacted worse than the others.<sup>28</sup> Rural America is one such example. Several rural community hospitals are already in fiscal crisis and the COVID-19 pandemic has exposed the vulnerability of these institutions that serve 20% of the US Populations.<sup>29</sup> While metropolitan cities across the United States received the media attention, they also received most of the initial

testing reagents and medical supplies.<sup>29</sup> This heavily handicapped smaller community hospitals and underserved populations forcing creative partnerships to address not only supply chain issues, testing constraints, but also accurate molecular diagnosis of COVID-19. The University partnership with a local Regional hospital has responded and provided an opportunity to the rural community in Western Pennsylvania of its unique challenges with unprecedented speed. The collaboration is using available technologies without having to compromise on the sensitivity and specificity of detection thus contributing to the global effort in different ways. The idea to partner was conceived in March 2020 between IUP and IRMC. IRMC reached out to IUP with hopes of developing regular testing to circumvent some of the problems associated with COVID-19 test services and supply chain issues. The contagious nature of the virus with both symptomatic and asymptomatic transmission, coupled with shortage of resources including test kits and viral transport medium, impacted many hospital treatments services and diminished critical patient care and surgical activity. This led to financial constraints in many hospitals, including IRMC in rural America. Thus, there was a pressing need for a low-cost, rapid, and sensitive detection protocol that was easily accessible locally. Moreover, lack of testing facilities in rural areas along with avoidance have been implicated in the slow progression of disease outbreak. A collaboration like ours between university and a rural community hospital should help to focus and address testing constraints and shelter rural communities from further COVID-19 transmission and outbreak.

## CONCLUSION

In this study, we have shown the significance of in-house testing COVID-19 that circulated during the outbreak in a rural community in Western Pennsylvania through a hospital university partnership. We present for the first-time comprehensive diagnostic test results in the data-limited rural community. This strategy of using dry swabs without any medium coupled with low-volume analytical variables potentially ramped up an essential health response in this new outbreak. The newly developed optimized scalable protocol could be easily adapted for routine testing and high-fidelity screening of both symptomatic and asymptomatic people, with very good STAT and investments in pre-analytical and analytical variables. Our collaborative effort helped to test more than 10,000 specimens using dry swabs.

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