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Validation of Microchip Based RT-PCR ABC Test (InfA/B & COVID-19) in Clinical Samples

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Abstract

To contain the rapid and global spread of SARS-CoV-2, it is essential to develop an accurate and sensitive test system to address pandemic bottlenecks, simplified sample collection, and no sample prep. While meeting the demand of testing large populations, the miniaturized volume of assay reagents and offering rapid results is the need in such scenarios. Moreover, in view of the reports of co-infections and overlapping symptoms of Influenza caused by *Influenza* A or *Influenza* B, and COVID-19 caused by SARS-CoV-2, a test system with three targets can be supportive for accurate clinical diagnosis. In this presentation, we evaluated the performance of a test comprising Microchip RT-PCR Influenza and COVID-19 Detection System for identifying these three viral pathogens in nasal swabs and saliva specimens. A rapid and simplified total nucleic acid extraction method was developed and validated for the reliable, high-throughput simultaneous detection of respiratory viruses causing Influenza (type A and type B viruses) and COVID-19 (SARS-CoV-2 virus) using the microchip-based AriaDNA™ platform deriving the name ABC Test. The test system was evaluated using 81 nasal swab samples, 77 clinical saliva samples, 5 blind CAP reference samples, and RNA standards. The limit of detection (LoD) was assessed using SARS-CoV-2, *Influenza* A, and *Influenza* B RNA standards. The multiplex ABC Test microchip displayed LoD of 14 copies/μL for SARS-CoV-2 and approximately 26 copies/μL for *Influenza* A, and 140 copies/μL for *Influenza* B, respectively. The ABC Test offers rapid multiplex one-step RT-PCR in 32 minutes for 45 cycles as the miniaturized reaction of 1.2 μL offering a highly sensitive, robust, and accurate assay for the detection of *Influenza* A/B, and SARS-CoV-2.

Keywords

SARS-CoV-2, Influenza, Multiplex, Microchip PCR, Nasal Swab and Saliva

1. Introduction

To halt the rapid spread of COVID-19 through populations requires increased diagnostic testing [1] [2]. Moreover, increasing testing frequency and reducing reporting time have a greater effect on controlling transmission than increasing test sensitivity to break chains of infection and allow earlier quarantine of infected individuals [3]. In this endeavour, the one-step real-time reverse transcriptase PCR (RT-PCR) is the globally preferred method for its diagnosis [4] [5] [6] [7]. Addressing the pandemic supply bottlenecks, and widespread implementation of testing along with minimization of the PCR reagent volume, rapid test results, simplified sample handling, and offering a user-friendly test system, a miniaturized disposable microchip-based Real-time RT-PCR test system has emerged [8] [9] [10].

The nasopharyngeal (NP) swabs as the originally RT-PCR testing standard and subsequently accepted oropharyngeal swabs, mid-turbinate swabs, and nasal swabs suffered from testing large populations for SARS-CoV-2 virus [11] [12] [13] [14]. High cost, exacerbated supply chain pressure, specialized equipment, large volumes of reagents, specimen collection & processing, and the need for medical personnel in sample collection turned out as serious bottlenecks. As an adequate substitute for the NP swab sample type, saliva-based testing emerged as an accurate diagnostic tool [15] [16] [17] [18] [19]. Apart from offering similar sensitivity and specificity as NP swabs, the saliva sample offers self-collection of specimens and is non-invasive to the patient as a major advantage [9] [18] [20]. Moreover, test cost is also reduced by saliva-based tests by stripping the need for virus transport media and replacing the RNA extraction process by performing proteinase K and heat extraction [18] [21].

Additional diagnostic difficulties in SARS-CoV-2-infected patients were increased due to the presence of the *Influenza* A virus [22]. In addition, concurrent infections of SARS-CoV-2 with Influenza viruses influence the morbidity and mortality of patients with COVID-19 [22]-[27]. Moreover, the diagnosis turns out to be more complicated due to the overlapping symptoms among Inf A, Inf B, and COVID-19 [28]. Therefore, accurate and rapid identification of coinfection of *Influenza* A and B in COVID-19 cases is required [24].

In this study, a dual multiplex RT-PCR analysis system coined as the multiplex ABC Test to simultaneously detect SARS-CoV-2, *Influenza* A, and B viruses is presented as a cost-effective *Taq*Man chemistry reaction of 1.2 µL in a 30-microwell microchip. The one-step RT-PCR reaction employs the US CDC-recommended N1 primers and probe for detecting the nucleocapsid (N) gene of SARS-CoV-2, *Influenza* A (Inf A), and *Influenza* B (Inf B) genes paired with a human specimen control, HsRP. A 2-plex test system offers a throughput of 27 samples in addition to the controls using simplified saliva and NS swabs. The primers and probes of the test kits are preloaded and lyophilized in the individual microwells of the microchip performing 45 cycles of one-step RT-PCR in 32 minutes [8] [9] [10].

2. Materials & Methods

1) Procurement of reagents: The reagents used in these studies were prepared as follows:

a) PCR reagents: Primers & probes were supplied by Integrated DNA Technology Inc, USA (https://sg.idtdna.com/pages) according to 2019-nCoV CDC EUA Kit (Cat # 10006770). The primer-probe set to detect N1 region of the nucleocapsid (N) gene of SARS-CoV-2 virus, and the second primer-probe set to detect Human RNase P (HsRP) gene of human genome as an internal control were lyophilized in the microchip for COVID-19 detection. The primer-probe sets to detect the matrix (M1) gene of *Influenza* type A virus, and the nonstructural-2 (NS2) gene of *Influenza* type B virus were utilized in ABC Test microchip in addition to N1 and HsRP assays.

UltraPlex™ 1-Step ToughMix®, a 4× concentrated master mix for one-step qRT-PCR containing dATP, dCTP, dGTP, dTTP, magnesium, qScript XLT reverse transcriptase, RNase inhibitor protein and AccuStart II hot-start Taq DNA polymerase, catalogue # 95166-01K procured from Quanta Bio, MA, USA (https://www.quantabio.com/) was utilized as the TaqMan *Taq* polymerase enzyme premix for real-time PCR on the microchip.

b) Virus transport medium (VTM): The VTM-Phosphate Buffered Saline tubes Cat # E504-500 ML were obtained from VWR, USA (https://www.vwr.com/).

c) RNA storage solution: The RNA storage solution Cat # AM7001 was obtained from Thermo Fisher, USA (https://www.thermofisher.cn/cn/zh/home.html).

2) Preparation of positive RNA controls: As a positive control in all experiments, *in vitro* transcribed RNA encapsulated in a protective protein coat, Armored RNA Quant that includes the viral sequences for the N1 RNA of SARS-CoV-2, 1×10^{11} copies/mL, Cat # 52036, and HsRP Armored RNA Cat # 52031, 1×10^{11} copies/mL, used as an alternative template to the human housekeeping gene (HsRP) and Armored RNA Quant Influenza 1×10^{6} copies/ml, Cat # 52013 was obtained from Asuragen Inc., Austin, TX, USA (https://asuragen.com/). Additional standards were Quantitative Genomic RNA from *Influenza* A virus (H1N1) strain: A/Virginia/ATCC1/2009, 1.8×10^{5} copies/μL, Cat # VR-1736DQ, and Quantitative Genomic RNA from *Influenza* B virus strain: B/Wisconsin/1/2010, 9.8×10^{5} copies/μL, Cat # VR-1883DQ was obtained from ATCC, Manassas, VA, USA (https://www.atcc.org/).

3) Disposable pre-filled microchips: The disposable pre-filled microchips with 30 microwells (6 columns × 5 rows) manufactured from aluminum sheets by metal stamping technology and coated with surface modifiers. This coating imparts hydrophilicity to the microwells of 1.2 μL capacity and hydrophobicity to the upper surface of the microchips. The empty microchips were then filled with a 1.2 μL solution of primers and probes along with stabilizing agents using a OT2 robotic workstation from Opentrons, USA (https://opentrons.com/) in a predefined layout (**Figure 1(a)** and **Figure 1(b)**). The prefilled microchips were then lyophilized by Lumex Instruments, Canada using a SJIA-10N Lyophilizer of

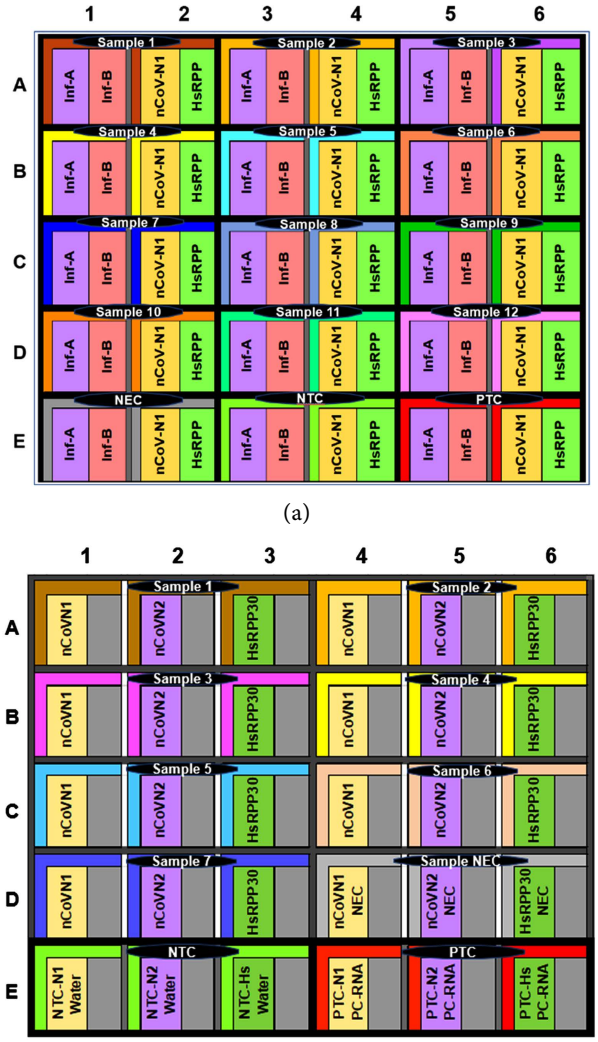

Figure 1. Microchip configuration. (a) Inf A (*Influenza* A), Inf B (*Influenza* B), nCoV-N1 (SARS-CoV-2), HsRP (Human specimen), NEC (negative extraction control), NTC (negative template control), PTC (positive template control). (b) nCoV-N1 (SARS-CoV-2), nCoV-N2 (SARS-CoV-2), HsRP (Human specimen), NEC (negative extraction control).

Ningbo Shuangjia Instrument Co., Ltd, China (http://www.shuangjiayiqi.com/) and each microchip was individually packaged in a package with desiccant.

4) Microchip-based one-step RT-PCR:

The mix of the sample and master mix for the RT-PCR assays was prepared as UltraPlex™ 1-Step ToughMix®: viral RNA: ddH_2O in the volumetric ratio of 1:1:2. To prepare Negative Template Control (NTC), viral RNA was replaced with ddH_2O or RNA Storage Solution. To prepare Positive Template Control (PTC), viral RNA was replaced with *in vitro* transcribed SARS-CoV-2 RNA (1×10^4 copies/μL), *Influenza* A and *Influenza* B (1×10^4 copies/μL), mixed with HsRP RNA (1×10^4 copies/μL).

Real-time RT-PCR was performed on the microchip-based PCR analyzer AriaDNA™ (https://www.lumexinstruments.com/) using manufacturer's soft-

ware to control the instrument and obtain PCR results following the instruction for use procedure. Before the addition of the reaction mix in the microchip, 620 µL of silicone oil layer was added for the purpose of overlaying the reaction mix to contain evaporation of the reagents during processing and thermocycling. The mix of sample and master mix was quickly followed by pipetting under the oil layer.

Each sample and control was loaded into the microwells containing SARS-CoV-2 targets (N1) and human sample control (Hs) primer-probes with 1.2 µL/microwell. Reactions were carried out on the AriaDNA™ PCR analyzer as one-step RT-PCR and run as 3 replicates (n = 3), if not stated otherwise.

Thermal protocol for COVID-19 test was as follows: a reverse transcription step at 50˚C for 900 s, followed by a denaturing step at 95˚C for 120 s and 45 cycles of 95˚C for 3 s followed by extension and signal recording at 55˚C for 30 s. For ABC Test, thermal protocol was as follows: a reverse transcription step at 50˚C for 600 s, followed by a denaturing step at 95˚C for 120 s and 45 cycles of 95˚C for 3 s followed by extension and signal recording at 55˚C for 30 s.

The Ct values were determined as a second derivative maximum (SDM) once fluorescence passed an auto-set SDM threshold. The SDM serves as an automated alternative but manually tweakable threshold setting and confers flexibility of the software on the instrument. SDM value is reported when an amplitude of the fluorescent signal and an amplitude of its first derivative are both above the pre-set thresholds. Those threshold values can be adjusted within AriaDNA™ software and were set at 150 and 50 arbitrary units, respectively. A sample was reported positive for SARS-CoV-2, *Influenza* A or *Influenza* B if either N1, Inf A or Inf B viral targets were detected with Ct < 40 passing data quality control. Negative samples required the internal HsRP control to be detected with Ct < 38.

5) Comparison of sensitivity and LoD of SARS-CoV-2 (N1) PCR: The sensitivity and limit of detection (LoD) of the RT-PCR was performed using low number of copies (6.25 copy/µL) of N1-RNA in the presence of higher number of copies of HsRP-RNA in comparison to control. Comparative Ct values were determined along with fluorescence intensities.

6) Test samples: A total of 81 nasal swab samples, 77 clinical saliva samples, RNA standards, and 5 blind CAP (College of American Pathologists, USA) reference samples were obtained to evaluate the ABC test system.

a) Sample collection:

- **Nasal or throat samples**: For taking the nasal or throat sample, a sterile single-use cotton swab was gently brushed inside the nose or mouth of the subject by the medical technician and the swab was inserted in VTM containing tube.
- **Saliva samples:** Patient samples were collected in 50 mL sterile centrifuge tubes and sampled no sooner than a half hour after eating or drinking. After collecting a minimum 50 µL of the sample, the lid of the collection container was securely replaced. Outer surface of the container was sterilized with 70%

alcohol and placed the sample in a secondary container. The test samples were analyzed within 6 hours or kept refrigerated at 2˚C - 8˚C for up to 3 days, or −80˚C for long-term storage, and eventually thawed on ice for testing.

b) Sample extraction

i) Magnetic bead-based extraction of RNA: The RNA from the nasal swabs in VTM samples, magnetic bead-based extraction was carried using the Nucleic acid extraction kit, Cat # AU17011 from Bioteke Corp Ltd, China (https://www.bioteke.com/). Manufacturer's instructions were followed to extract RNA.

ii) Spin column-based extraction of RNA: The nasal swabs, and saliva samples of COVID were processed through Norgen Biotek's (https://norgenbiotek.com/) extraction kit Cat # 17200. The samples other than saliva were processed through the following steps:

- **Cell lysate preparation from nasal or throat swabs:** The nasal or throat sample were followed by taking 300 µl of the nasal or throat swab sample-VTM and added to 1.5 mL (or 2.0 mL) RNase-free microcentrifuge tube, excluding extraction control tubes. Then 600 µL of Buffer RL from the sample preparation kit was applied to each microcentrifuge tube containing the patient nasal-VTM sample or saliva-VTM sample.
- **NEC and PEC**: To the NEC and PEC control tubes containing 100 µL of negative patient saliva 600 µL of Buffer RL was added.

- All the tubes prepared above, were gently vortexed after closing cap and incubated the tubes for 5 minutes at room temperature.
- Following incubation, the PEC tube was spiked with 10 µL of 1×10^4 copies of the standard RNA and gently mixed it by vortexing.
- After adding an equal volume of 70% ethanol to all the lysate volumes, the tubes were vortexed for 3 sec to mix the contents.

- **Kit manufacturer's instructions:** All the steps on Binding RNA to the column, Column wash, RNA elution, were followed from the kit manufacturer's instructions.
- **Storage of RNA**: The purified RNA sample was either used right away for PCR or stored at −20˚C for 72 hours or at −70˚C for long term storage.

iii) Extraction-free saliva sample processing: For saliva samples, nucleic acid clean up was performed with the proteinase K (New England Biolabs (https://www.neb.com, cat # P8107S) using a heat block as follows:

- After adding 6.25 µL (20 mg/mL) of proteinase K to homogenized saliva, contents were mixed by vortexing each saliva sample immediately after addition of proteinase K. It was followed by incubation at 65˚C for 5 minutes and inactivation of the proteinase K, ramp to 95˚C for 5 minutes and hold at 95˚C for 5 minutes on incubator heat block.
- 2.5 µL of Saliva sample processed in the previous step was mixed with 2.0 µL of nuclease free water and 1.5 µL QuantBio Ultraplex 1-Step ToughMix in a 250 µL centrifuge tube and micro-centrifuged for 3 seconds. The mixture was

pipetted as 1.2 μL aliquots into individual microreactors of the lyophilized microchips following the layout.

7) Molecular testing: A reverse transcription polymerase chain reaction (RT-PCR) based on primer-probe sets for the SARS-CoV-2 N1, Inf A, Inf B genes (N1 and Inf A, Inf B targets) and human control ribonuclease P HsRP was validated for clinical use following the regulatory requirements of CLIA and the Federal Drug Administration's Emergency Use Authorization criteria.

8) Analysis: Following the Microchip RT-PCR COVID-19 Detection Kit instructions for use, the analysis of the data was performed. Calculations on the number of copies of the RNA, instrument detection limit (IDL), and Student's t determination of LoD as RNA number of copies is as follows:

a) Instrument detection limit (IDL): Minimum detectable concentration of a fluorescent dye (Cmin) was calculated as 3 times the standard deviation of the background (Std dev Background) multiplied by the concentration of the 6FAM (C6Fam), divided by the difference of the 6FAM intensity and the background intensity (I 6Fam - I background).

$$\text{Cmin} = \frac{3 * \text{Std dev Background} * \text{C6Fam}}{\text{I 6Fam} - \text{I background}}$$

Cmin was estimated to be (3 × 7.8 × 100 nM)/(3341 − 2596) = 3.14 nM.

IDL at 95% confidence level (IDL95) was estimated using the formula $t(n-1, 1-\propto = 0.95) * \text{STD EV}$, where $t = 2.3533$ at $n = 3$. IDL95 was found to be 18.4 nM for FAM dye.

b) The calculations on LoD as RNA number of copies: LoD was calculated as RNA copies per reaction using the following formula:

$$\begin{aligned}&\#\text{RNA copies per reaction}\left[\text{copies}\right]\\&= \frac{\text{RNA Spiked}\left[\frac{\text{cp}}{\text{uL}}\right] * \text{Volume\_spiked}\left[\text{uL}\right]}{\text{Volume Lysis}\left[\text{uL}\right]} * \frac{\text{Volume}_{\text{RNA}_{\text{sample}}}\left[\text{uL}\right]}{\text{Total}_{\text{volume}}\left[\text{uL}\right]}\\&* \text{Microreactor volume}\left[\text{uL}\right]\end{aligned}$$

where,

RNA Spiked is the RNA concentration of stock standard;

Volume Spiked is the volume of stock standard added;

Volume digested is the Volume Lysis of digested saliva with proteinase K after sample prep;

Volume RNA sample is the aliquot of the Volume Digested to be used in the PCR Mix;

Total Volume is the resulting solution of the RNA Sample, Nuclease free water and the Master mix;

Microreactor volume is 1.2 μL aliquot of the total volume used in the microwells.

Assuming

LoD expressed as number of RNA copies per reaction was found to be

$$\frac{125\left[\frac{cp}{uL}\right]*6.25[uL]}{62.5[uL]}*\frac{2.5[uL]}{6[uL]}*1.2[uL]=5.21\left[\frac{cp}{uL}\right]$$

3. Results and Discussion

In view of the benefits offered by the miniaturized reaction volume offered by the microchip, the ABC test system was validated in terms of sensitivity and specificity with clinical samples using simplified processing of saliva samples with proteinase K and nasal swabs using spin columns.

Analytical sensitivity (LoD): LoD studies determine the lowest detectable concentration of 2019-nCoV at which approximately 95% of all (true positive) replicates test positive [29]. Accordingly, the LoD was determined by limiting dilution studies using characterized samples. The analytical sensitivity of the N1 assays of the Microchip RT-PCR COVID-19 Detection Kit was determined in LoD studies. Since no quantified virus isolates of the 2019-nCoV were available at the time of this study, the assays designed for detection of the 2019-nCoV RNA were tested with characterized stocks of *in vitro* transcribed RNA encapsulated in a protective protein coat (Armored RNA Quant SARS-CoV-2 Panel, Cat. # 52036) of known titer (RNA copies/µL) spiked into a diluent consisting of a suspension of human saliva to mimic clinical specimen (**Table 1** and **Table 2**, **Figure 2** and **Figure 3**).

LoD for processed saliva samples: The proteinase K treated saliva method has since been widely adopted and used in a large number of certified labs [30]. This method has also been modified replacing the use of proteinase K with a temperature gradient by heating at 95˚C/75˚C [31]. Saliva swabs have been reported having high sensitivity and specificity for the detection of Influenza virus by the Xpert Xpress Flu/RSV test with a high overall agreement and Ct correlation with nasopharyngeal specimens [32].

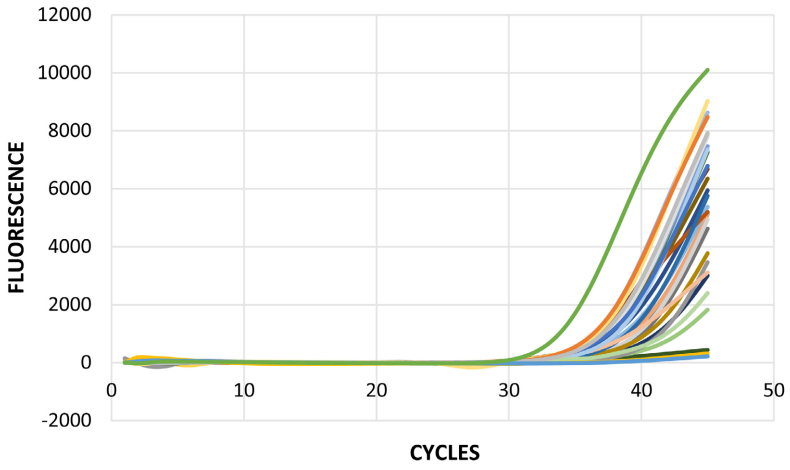

Figure 2. Sars-CoV-2 12.5 copies/µL, LOD confirmation in saliva with 27 samples, NTC, NEC, and PEC. Saliva samples were performed with LDT and not with SalivaDirect™, optimized to be used with AriaDNA.

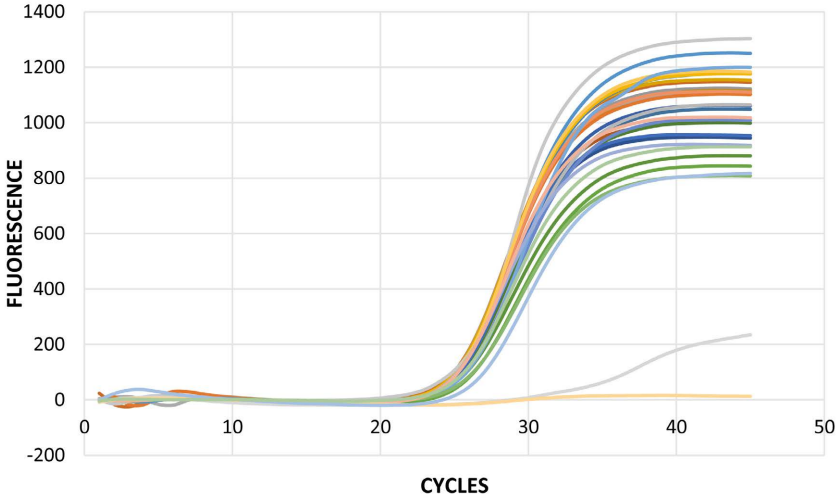

Figure 3. HsRP, LOD confirmation in Saliva with 27 samples, NTC, NEC, and PEC. Saliva samples were performed with LDT and not with SalivaDirect™, optimized to be used with AriaDNA.

Table 1. Threshold cycle values (Ct) of two-fold dilution series for SARS-CoV-2 in saliva samples.

Copies/μL	Ct1	Ct2	Ct3	Ct Ave	Std Dev
100.0	32.6	31.46	30.6	31.55	1.00
50.0	30.97	32.52	32.08	31.86	0.80
25.0	32.67	32.03	32.47	32.39	0.33
12.5	34.07	35.29	34.62	34.66	0.61
6.25	N/A	34.5	34.95	34.720	0.22

Table 2. Threshold cycle values (Ct) of ten-fold dilution series for ABC test of standard RNA samples.

Copies/μL	10,000	1000	100	10
Sars-CoV-2 Ct	28.08	29.67	29.74	30.51
Standard Deviation	1.07	0.41	0.20	0.08
Copies/μL	18000	1800	180	18
***Influenza* A Ct**	22.43	25.58	28.27	30.98
Standard Deviation	0.65	0.37	0.07	0.15
Copies/μL	98,000	9800	980	98
***Influenza* B Ct**	20.95	24.22	26.87	30.51
Standard Deviation	0.35	0.40	0.09	0.08
Copies/μL	10,000	1000	100	10
HsRP Ct	27.60	28.08	29.74	30.51
Standard Deviation	0.99	0.08	0.20	0.08

In the present work, saliva samples were treated with proteinase K. A preliminary LoD for each assay was determined testing in triplicate (n = 3) samples of RNA purified using each extraction method, SalivaDirect™ [18] and in house Laboratory Developed Test (LDT). The approximate LoD was identified by extracting and testing 10-fold serial dilutions of characterized stocks of *in vitro* transcribed Armored RNA Quant SARS-CoV-2 Panel (Table 3). A confirmation of the LoD was determined using 2-fold serial dilution of RNA samples with >20 extracted replicates (n = 20). The LoD was calculated as the lowest concentration where ≥95% (19/20) of the replicates were positive (Table 1 and Table 2).

The results demonstrated 15 copies per microliter of SARS-CoV-2 in each tube of processed saliva. However, the number of copies in reaction (per well of the microchip) was much lower as demonstrated in the sample calculation section.

In the LoD studies of SARS-CoV-2, the lysate in the tubes was spiked to have a final concentration 12.5 copies/μL in the reaction. The microchips ran an LoD of 12.5 copies/μL and the confirmation reflected 26/27 = 96.2% ± 1.73% positive agreement rate whereas, the sensitivity and specificity were conveyed as follows (Figure 2 and Figure 3, Table 3):

Positive percent agreement (sensitivity) = 26/27 = 96.2%;

Negative percent agreement (selectivity) = 12/12 = 100%.

LoD nasal swabs versus saliva samples: Nasal swab samples produced similar results for ABC Test, with lower LoD for SARS-CoV-2, *Influenza* A and *Influenza* B. Saliva processed samples yielded approximately 248 copies/μL for *Influenza* A, and 132 copies/μL for *Influenza* B per sample. The *Influenza* A and B RNA had extracted better in a high salt content than saliva alone (Figure 4). ABC spiking solution was diluted with Thermo Fisher nuclease free RNA storage solution. However, the ABC Test displayed less interference when samples were diluted with VTM 50 μL spiking solution than 50 μL saliva spiked with 5 μL of ten times more concentrated ABC. Mixing ABC spiked 50 μl RNA storage solution with the 300 μL of VTM, and the nasal swab also resulted better extraction than saliva without dilution. [Data not presented due to lack of fluorescence with 5 μL spikes of 10^2 or less]. Dilution of the saliva to reduce viscosity and homogeneity improves recoveries of spiked RNA.

Table 3. Threshold cycle values (Ct) of ten-fold dilution series for ABC test of nasal swab samples.

Copies/μL	10,000	1000	100	10
SARS-CoV-2-CoV-2 Ct	27.27	31.86	33.95	45
Copies/μL	2314	231.4	23.14	2.314
***Influenza* A Ct**	32.48	33.64	34.29	42.31
Copies/μL	12600	1260	126	12.6
***Influenza* B Ct**	30.93	31.03	32.12	34.4

Validation of the ABC Test: Due to non-availability of clinical samples for *Influenza* A and B, the spiked samples with *Orthomyxoviridae*, *Influenza A & B* viral RNA were used. The preliminary work demonstrated the successful working of the multiplex microchip for ABC Test (Figure 1(a)). Preliminary testing specificity and cross-reactivity of the RNA targets in the Inf-COVID chip demonstrates no cross-reactivity in the multiplex of *Influenza* A for H1N1 and H3N2. Furthermore, the ABC assay is non-cross-reactive in the 2-plex of (InfA/InfB) where Inf-A assay did not react with Inf-B RNA and vice versa and further with SASR-CoV-2 N1/HsRP (Figure 5 and Figure 6).

The test results for positive COVID were also confirmed with nasal swabs on the SARS-CoV single-plex microchip that offers testing of N1, N2, and HsRP for 7 samples (Figure 1(b)). The single-plex microchip test was validated, and representative PCR curves are shown in Figure 5 and Figure 7. A confirmation to verify reagents and equipment were not contaminated, was performed with SalivaDirect™ with a different PCR instrument [Data not presented].

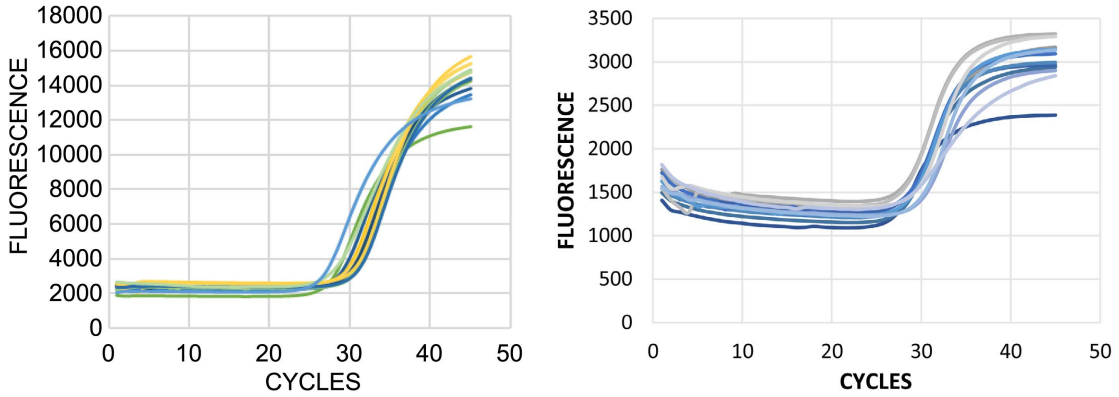

Figure 4. ABC Standards from Table 1. (a) 10-fold dilution of *Influenza* A RNA standard. (b) 10-fold dilution of *Influenza* B RNA standard. Saliva processed samples yielded approximately 248 copies/μL for *Influenza* A, and 132 copies/μL for *Influenza* B per sample.

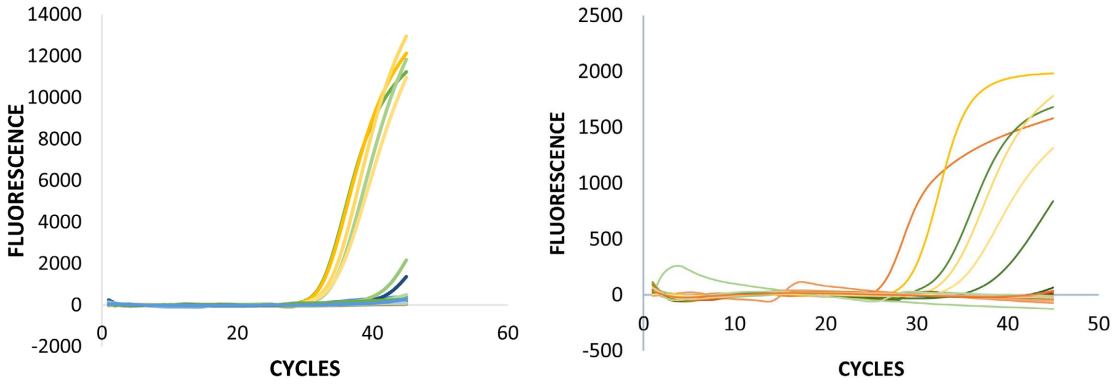

Figure 5. Saliva spiked (10-fold dilution starting at 10^3 copies/μL) multiplex: (a) *Influenza* A and SARS-CoV-2 N2. (b) *Influenza* B and HsRP30.

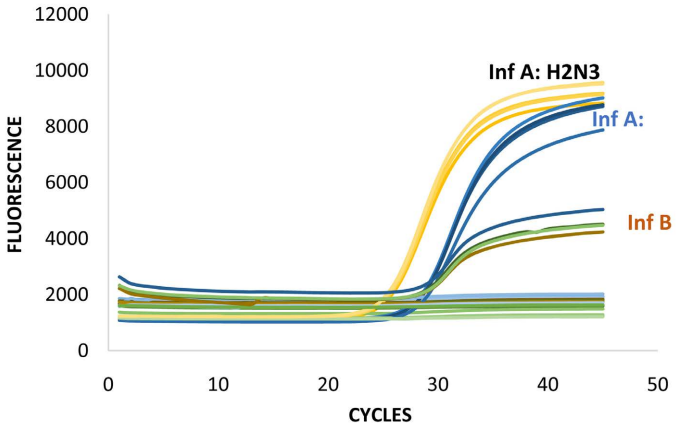

Figure 6. Preliminary testing specificity and cross reactivity of the RNA targets in the Inf-COVID chip demonstrates no-cross reactivity in the multiplex of *Influenza* A and *Influenza* B.

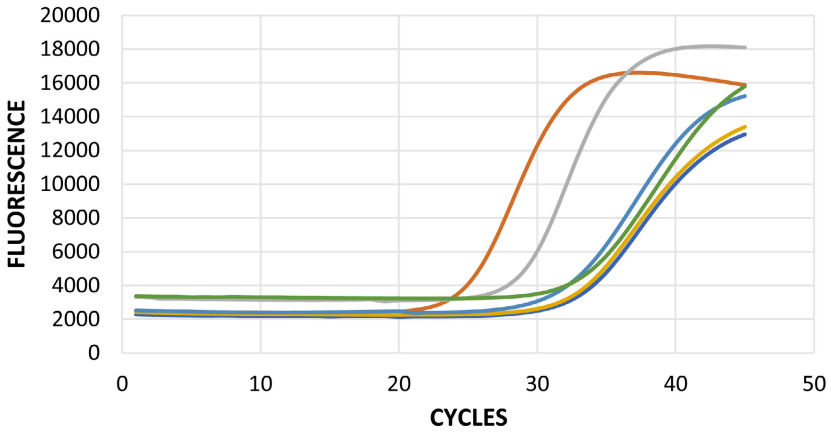

Figure 7. Single-plex NEC and principal investigator test positive on Nov 17, 2021, confirmation. The test results for positive Sars-CoV-2 (N2) were confirmed with nasal swabs on the single-plex microchip that offers testing of N1, N2, and HsRP for 7 samples.

Table 4. Confirmation.

Samples	Results	Confirmation	
		CAP	Abbott/Other
Inf A	1 out of 5 Positive	Passed kit #34911824	Confirmed
Inf B	0 out of 5 Positive	Passed kit #34911824	Confirmed
SARS-CoV-2 N	2 out of 5 Positive	Passed kit #35107854	Confirmed

4. Conclusions

The multiplex ABC Test microchip LoD was obtained to be 14 copies/μL SARS-CoV-2 (N1), 26 copies/μL for *Influenza* A, and 140 copies/μL for *Influenza* B RNA per sample for nasal swabs. Furthermore, the LoD of the SARS-CoV-2 assay was found to be lower than that of the reported LOD of a widely used dual multiplex real-time RT-PCR having an LoD of 50 copies/reaction and compara-

ble to the LoD of *Influenza* A/B with 100 - 200 copies/reaction nasopharyngeal swab clinical samples [27].

The microchip also demonstrated 3 to 10-fold minimization of PCR master mix use by reducing sample size. Spiked RNA of *Influenza* A (23.1 copies/reaction) and B (126 copies/reaction) and SARS-CoV-2 mixed together yielded sensitivity and selectivity > 95%. Although the calculations were reported per reaction, they can be easily calculated per sample volume as stated in the Sample Calculations Section. The first set of CAP samples of *Influenza* A, *Influenza* B, and COVID-19 passed blind performance checks (**Table 4**). The diagnostic approach presented here is a robust assay for RT-qPCR which otherwise can suffer from reagent shortages or false results in typical patients. High throughput of the assay has been achieved with magnetic bead sample extraction and purification where the highly pure form of streptavidin covalently coupled with 1 μm superparamagnetic particles were used. The beads capture biotin-labeled substrates including antigens, antibodies, and nucleic acids. Such RNA from the lysed samples is retrieved with biotin-streptavidin complex, washed, and then eluted. Such approaches have been developed for SARS-CoV-2 and are under development for Influenza. We are waiting for Influenza clinical samples to confirm the process.

We have submitted our application for an EUA # 210069 based on the CDC methodology for a multiplex ABC Test. In addition, we have shared our findings with other research groups to support the effort in combating the current pandemic.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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