Wide application of minimally processed saliva on multiple RT-PCR kits for SARS-CoV-2 detection in Indonesia

Caroline Mahendra¹,* , Maria M. M. Kaisar²,* , Suraj R. Vasandani¹,* , Sem Samuel Surja² , Enty Tjoa² , Febie Chriestya²,⁴ , Kathleen Irena Junusmin¹ , Tria A. Widowati² , Astrid Irwanto¹,³ , Soegianto Ali²,#

¹Nalagenetics Pte Ltd, Singapore, Singapore
²School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
³Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore
⁴Rumah Sakit Pendidikan & Pusat Penelitian Atma Jaya, Jakarta, Indonesia

*First authors - equally contributed

#corresponding author: Soegianto Ali, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia. Pluit Raya No. 2, Jakarta 14440, Indonesia. E-mail: soegianto.ali@atmajaya.ac.id

RUNNING TITLE: Saliva PCR for SARS-CoV-2

WORD COUNT: 3646

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
Wide application of minimally processed saliva on multiple RT-PCR kits for SARS-CoV-2 detection in Indonesia

Saliva as a sample matrix has been an attractive alternative for the detection of SARS-CoV-2. However, due to potential variability in collection and processing steps, it is recommended to evaluate a proposed workflow amongst the local population. Here, we aim to validate collection and treatment of human saliva as a direct specimen for RT-qPCR based detection of SARS-CoV-2 in Indonesia. We demonstrated that SARS-CoV-2 target genes were detected in saliva specimen and remained stable for five days refrigerated or room temperature storage. The method of processing saliva specimen described in this report is free from RNA-extraction step, thereby reduces cost, time, and manpower required for processing samples. The developed method was validated for use on three COVID-19 RT-PCR kits commercially available. Our developed method achieved 85% agreement rate when compared to paired nasopharyngeal and oropharyngeal swab specimens (NPOP). With the assistance of a specimen sampling device, QuickSpit™, collection was found to be more convenient for individuals and improved agreement rate to 90%.

Keywords: saliva; SARS-CoV-2; real-time PCR; Direct-PCR; stability; cost reduction
INTRODUCTION

COVID-19 case was first reported to the World Health Organization (WHO) on December 31, 2019 and was declared as pandemic on March 11, 2020 [1]. COVID-19 was caused by coronavirus, an enveloped virus with single stranded positive strand genomic RNA [2]. One of the modalities used to diagnose COVID-19 is nucleic acid amplification test (NAAT), including Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) which commonly targets the envelope (E), nucleocapsid (N), RNA-dependent RNA polymerase (RdRP), and spike (S) genes [3]. Specimens that could be used for NAAT includes those obtained from the upper and lower respiratory tracts and gastrointestinal tract [4]. Specimens commonly used from the upper respiratory tracts are nasopharyngeal and oropharyngeal (NPOP) swabs and samples obtained from the lower respiratory tract include bronchoalveolar lavage. Rate of detecting positive infection from bronchoalveolar lavage is superior when compared to that of NPOP swabs, however it is commonly obtained from inpatients with severe illness or undergoing mechanical ventilation [4]. Stool has also been used as a specimen for NAAT methods for covid detection including among children [5].

Collecting NPOP specimens require trained healthcare personnel and could induce aerosolization that increases risk of infection to the healthcare personnel. Adequate Personal Protective Equipment is needed, and specimen collection can only be done in designated sites to reduce the risk of transmission during the procedure. Indonesia is a very vast country with varying level of access to collection sites at medical facilities. If contact or suspected COVID-19 patients live away from the collection site, they might need to use public transport or go through certain distances to reach the center for NPOP collection [6]. This could increase risk of infection from COVID-19 suspects exposure to the general public.
Other studies have shown that saliva can serve as an alternative specimen for NAAT-based SARS-CoV-2 testing [7,8]. Its non-invasive nature reduces the level of discomfort when sampling, does not induce production of aerosols and does not require trained healthcare provider, which could allow for flexibility in various collection sites including at-home. Although there is reduction in sensitivity of detecting of SARS-CoV-2 from saliva samples, its specificity remained at par with NPOP, suggesting that saliva is still a reliable specimen [9–11].

The standard protocol for detecting SARS-CoV-2 using RT-qPCR method from NPOP needs various consumables. The NPOP swab should be kept in Viral Transport Medium (VTM) for transportation from swab location to the laboratory. In the laboratory, the VTM containing NPOP specimens are extracted to isolate purified viral RNA. This step generally utilizes a commercial RNA extraction kit and could take up to 1 – 1.5 hours to complete. Purified viral RNA then will be used as template for RT-qPCR amplification which takes 2 – 3 hours to set up and complete. In total, the whole procedure could take 3 – 4.5 hours from sample to result.

Other studies have reported a comparable result of conducting RT-qPCR directly from NPOP without the RNA extraction step [12,13]. Direct PCR omits the need of RNA extraction kit and reduce the turnover time by 1 – 1.5 hour. Currently in Indonesia, most RNA extraction kits are imported, and at a certain time during this pandemic, extraction kits can be scarce.

Considering the above-mentioned possibilities, we tested and validated detecting SARS-CoV-2 by direct RT-qPCR from minimally processed saliva specimen. The validated method would be more convenient for patients, safer for health workers and could reduce the time and the cost of current RT-qPCR test for detection of COVID-19 infection.
MATERIALS AND METHODS

Ethical Clearance

The collection of clinical samples, NPOP swabs and saliva samples, was approved by the Institutional Review Board of the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia (No:16/11/KEP-FKIKUAJ/2020).

Study Recruitment

Recruitment for study participants was done in collaboration with several SARS-CoV-2 testing sites or home service patients. Participated patients were verbally informed about the study and the procedure involved. Written informed consent, along with symptoms- if any, was obtained from all participants prior to sample collection. Patients who tested positive up to 14-days prior to sample collection, were symptomatic or were in close contact with a known positive patient were qualified to take part in this study. Critically ill, unconscious and/or intubated patients were excluded from this study.

Specimen Collection

The NPOP swab was collected from each patient by a trained medical professional and put into the same VTM tube. Prior to saliva sample collection, patients are required to satisfy a 30-minute fasting period during which they are to refrain from eating, drinking, smoking, tooth brushing, using mouthwash, etc. A 5 mL of unstimulated saliva is collected into a 50 mL tube, without the addition of buffers or any other stabilizing medium. Samples were then kept on ice during
transport to the laboratory. Samples were processed in an enhanced Biosafety Laboratory level 2 at the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia.

**Viral RNA Extraction**

Viral RNA was extracted with QIAmp viral RNA Mini kit following instructions provided by manufacturer (Catalog # 52906, Qiagen, Hilden Germany). In brief, 140µL of NPOP or saliva in sample is mixed with lysis buffer, bound to silica membrane present in the spin column, washed twice, and eluted (60µL) as pure RNA.

**Optimization of RNA-Extraction-free treatment**

RNA-extraction-free of saliva samples was performed in six different procedures as compared to regular viral RNA extracted-method from NPOP samples along with viral RNA extracted from saliva samples. In brief, 100µL of saliva samples were vortexed, aliquoted into 1.5mL tubes and were subjected to the different treatments before being used as PCR templates. The different procedures for optimizing the RNA-extraction-free treatment show in Figure 1A. The scheme was also done on both negative samples to avoid false positive results (Supplementary Information 2).

**Stability**

We determined the stability of saliva as a matrix for detection of SARS-CoV-2 from 14 saliva samples. 100µL of each sample were aliquoted into different tubes and stored either at room temperature or inside a refrigerator. Samples stored at room temperature were tested daily for
five days while samples stored in the refrigerator were tested on days three through five. RNA-extraction free treatment of saliva was done post incubation period before subjecting samples to RT-qPCR with reference kit.

**RNA-Extraction-free treatment of saliva collected samples**

After the optimizing the methods and stability, subsequent saliva specimens arriving in the laboratory were stored in refrigerator at approximately 4°C and processed within 5 days of collection date. Collection tubes containing saliva specimens were homogenized using a vortex-mixer for 10 seconds. 100µL saliva from the collection tube was transferred into microtubes and heated at 95°C for 10 minutes. Microtube containing heat-treated saliva was removed from the heat-block and stored in refrigerator at approximately 4°C for a maximum of 2 hours before being used as a template for downstream PCR reaction.

**Viral Nucleic Acid Detection**

Da An Gene’s Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing) (Catalog # DA-930) was used as the reference nucleic acid detection kit in this study. The RT-qPCR mastermix was prepared following manufacturer’s recommended instruction - 17µL of PCR reaction solution A and 3µL of PCR reaction solution B for each reaction. Upon template addition, strip tubes were briefly spun down to ensure that all liquid is positioned at the bottom of the tube. Thermocycling conditions are as follows: 15 minutes at 50°C, 15 minutes at 95°C, and 45 cycles of 15 seconds at 94°C and 45 seconds at 55°C. Samples were considered positive if Ct values for N-gene (FAM) and ORF1ab (VIC) were not more than
40 and the curve obtained showed obvious amplification with a form of a typical “S” shape. Samples that had single-channel amplification were regarded as invalid.

We validated the compatibility of this method with other commercial kits following instructions provided by each manufacturer. The internal controls used were added as additional template into the PCR master mix. For kits that required internal control to be added into samples prior to extraction, the step was modified to add internal control into the PCR master mix at 0.1x of its recommended volume. Details of targeted genes, internal control used, limit of the detection, thermocycle setting, number of cycles, cycle threshold cut off, master mixture, and RNA template volume was summarized in Supplementary Table 1 (S1).

The amplification, detection and analysis were performed using the CFX real-time detection system (Bio-Rad laboratories). Negative and positive controls were included in each PCR run. Cycle threshold (Ct) value results were analyzed using Bio-Rad CFX software (Maestro). The Ct-value results represent the amplification cycle in which the level of fluorescence signal exceeds the background fluorescence, reflecting the specific SARS-CoV-2 RNA load in the sample tested.

QuickSpit™ Prototyping

To ease sample collection, we conducted user research to survey usability of different prototypes. Target users were individuals of the following demographics: children, seniors and sick unable to travel. The factors in consideration for the survey were: clarity of instructions,
shape and size of mouthpiece, easiness to hold, specimen volume and hygiene. A package of 5mL collection vial, three different mouthpiece types (straw, triangle-shaped, round-shaped) and hardcopy of instructions for collection of 1.5mL saliva were distributed to consented respondents and their feedbacks were collected through an electronic response survey.

10 respondents were collected to cover various age groups and demographics (Supplementary Information S4A). With majority referring to common preferences as displayed in Supplementary Information S4B, QuickSpit™ was designed to have the following features: product insert containing step-by-step illustrations to assist self-collection, a wide-opening mouthpiece that attaches onto the collection vial, a collection vial of 5mL in size to facilitate collection of 0.5 – 1 mL of saliva specimen.

Data Management and Statistical Analysis

Collected respondent’s information and RT-qPCR data were stored in an MS Excel database with restricted sharing to authors only. Paired t-test were performed to compare the means of Ct value between different saliva sample treatments and mean difference between the varying storage conditions. Null hypothesis stated there is no difference in means of Ct value across the tested conditions. The data was presented as an odd ratio with 95% confidence interval (CI). Differences with a p-value < 0.05 were considered to be statistically significant. Statistical analysis and visualization of data were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA USA) and MS Excel for Windows.
RESULTS

*Transitioning from NPOP extracts to Saliva extracts for detection of SARS-CoV-2 by PCR*

We first sought to validate saliva as a specimen suitable for detection of SARS-CoV-2 by RT-qPCR. Respondents were collected for their NPOP swabs within one viral transport media (VTM) and saliva in separate tube. A total of 116 paired samples were collected and extracted using QIAamp Viral RNA Extraction kit and subjected for SARS-CoV-2 detection using 2019-nCoV RNA Detection Kit from Da An Gene Co (Table 1) targeting ORF1ab and N gene. We found an 89.3% overall agreement between samples extracted from saliva and NPOP, consisting of 84.5% positive percent agreement (PPA) and 100% negative percent agreement (NPA). There were eleven specimens that tested negative on saliva but positive on NPOP. Although this is in conjunction to previous reports that some variation exists between specimen types [10,11,14], our results still confirms that saliva is a viable alternative for specimen collection.

Out of the 116 samples collected, we found one sample that was invalid on both sample types upon detection via RT-qPCR. The invalid rate for testing saliva was 4% higher than NPOP specimens (invalid samples in saliva = 7%; NPOP = 3%). This could arise from carryover of interfering substances from the oral cavity during collection, which was observed in some samples that were colored, viscous and/or particulate.

*Development of method enabling Direct-PCR Detection of SARS-CoV-2*

To streamline the process of sample treatment for downstream PCR application, we explored several methods of treatment on saliva samples. This included saliva undergoing heating,
addition of Proteinase K and concentrating by centrifugation paired with RNA extracts from NPOP and saliva as templates for RT-qPCR reaction (Figure 1A). We found all methods of treatment on saliva resulted in the same qualitative outcome on positive specimens and comparable Ct for the two virus target genes (Figure 1B). The scheme was also done on both negative samples (Supplementary Information 2) to avoid false positive results.

Given the need for an affordable and scalable yet effective method, we developed a sample treatment that only involved heating of saliva. Paired t-test was performed between Ct values obtained from positive samples that were extracted from NPOP and heat treatment alone of saliva. There was no significant difference in detection of N-gene (p-value = 0.102) and ORF1ab (p-value = 0.107), demonstrating effectiveness of SARS-CoV-2 detection from saliva treatment as compared to RNA extract from NPOP. This method also confirmed qualitative results on negative specimens (Supplementary Information 2).

To evaluate the sensitivity of this method on detection of SARS-CoV-2, we compared the Ct value obtained for 55 samples that tested positive in saliva specimens. Our results generated linear regression of strong positive correlation in Ct value for N-gene (coefficient = 1.00, R² = 0.929) and ORF1ab (coefficient = 0.966, R² = 0.837), demonstrating that the heat incubation method is effective for treatment of saliva for diagnostics (Figure 2).

**SARS-CoV-2 in Saliva are stable for storage at 4°C and room temperature (RT)**

To determine the storage condition for specimens that maintain effective treatment and detection of SARS-CoV-2, we stored 14 saliva specimens at refrigerator (2-8°C) and room temperature in the laboratory (24-27°C) for 5 days. Each saliva was then subjected to heat incubation followed
by RT-qPCR detection at selected time points during the period of storage. We found that detection of both target genes remained stable for 5 days at both storage conditions for samples from low to high viral load with initial Ct ranging from 14 to 35 (Figure 3, Supplementary Information 3).

Paired t-test was performed to compare the mean differences between the varying storage conditions, where the null hypothesis states there is no difference in means of Ct value. On detection of N-gene, we found no significant difference in Ct values upon storage for 5 days at 4°C (ΔCt = 0.52, p-value = 0.262) and room temperature (ΔCt = 1.00, p-value = 0.066). There was no significant difference in the detection of N-gene between storing samples at both temperatures (p-value = 0.341). ORF1ab detection slightly improved upon storing for a period of 5 days, as seen in the decrease in Ct value at 4°C (ΔCt = -0.386, p-value = 0.295) and room temperature (ΔCt = -0.281, p-value = 0.559), although there was no significant difference between the two temperatures (p-value = 0.671).

**Validation of method for saliva-treatment**

To validate this method, a total of 118 patients were recruited with written informed consent for collection of NPOP and saliva at the same time point by healthcare workers (Table 2). NPOP were subjected to QIAamp Viral RNA Extraction while saliva specimens were treated under the RNA-Extraction-free method, and both applied as template for subsequent PCR detection. We found the overall percent agreement between NPOP-extract and saliva-treatment samples to be 85.4%, with 79.2% PPA and 100% NPA. There were fifteen specimens that tested different results from one sample type against the other. This was expected due to variation in sample
types but did not rule out the validity of this method of saliva treatment. As seen previously, the rate of invalid results from saliva were approximately 4% higher than NPOP specimens.

**Saliva heat treatment is versatile with a number of commercial COVID-19 RT-qPCR kits**

To assess the versatility of method for saliva treatment, we subjected the heated-saliva for detection using other commercial COVID-19 RT-qPCR kits available and compared the results to the reference kit that this method of treatment was developed on, Da An Gene Co. Other commercial kits had agreements and estimated sensitivity ranging from 70 – 100% (Table 3). This variation could exist due to differences in each kit’s characteristics as displayed in Supplementary Information 1. Compatibility of a kit with templates added from heated saliva were considered for kits generating high valid and agreement rates.

Two out of the eight commercial kits tested, Maccura SARS-CoV-2 Fluorescent PCR Kit and Fosun COVID-19 RT-PCR Detection Kit, had above 90% valid and 100% agreements with results obtained from the reference kit, Da An. Our results demonstrated wide applicability of this saliva heat treatment for adoption with other existing workflows.

**Implementation of QuickSpit™ as collection and treatment method for SARS-CoV-2 diagnostics**

To better assist with the specimen collection process, we utilized QuickSpit™ Collection Kit for easy and safe sampling of human saliva. This was considered from conducting a user research on preferences and concerns of saliva sampling. The study recruitment was continued at multiple collaborating sites using QuickSpit™ Collection kit. 69 of paired NPOP and QuickSpit™
samples were collected and processed to evaluate the percent agreements between methods (Table 4). QuickSpit™ improved the agreement between heated-saliva and extracted-NPOP methods of SARS-CoV-2 treatment before PCR detection, where 90% overall agreement was achieved with PPA at 85% and NPA 100%. Overall improvement was seen at an increase of 5% for all agreements from previous validation, demonstrating the effectiveness of implementing QuickSpit™ as a method for saliva collection. Moreover, the respondents perceived it to be more convenient and easier to use. The use of a collection device improved the quality of saliva collected that were evidently less viscous, less particulate and uniformly clear-colored which could lead to less accumulation of potential inhibitors.

**DISCUSSION**

In this study, we validated the application of human saliva as a candidate specimen for collection and specimen treatment in detection of SARS-CoV-2, achieving 90% agreement with the current conventional method using NPOP specimen. We also demonstrated that saliva remained stable for storage up to 5 days in refrigerator (2 – 8°C) and room temperature (24-27°C), and that the candidate method of treatment is versatile for a number of commercial COVID-19 qPCR kits available in Indonesia. Numerous references have reported the use of human saliva as an attractive specimen for detection of SARS-CoV-2 infection for its practicality in sampling and processing [8,9,14–18]. Collection of saliva allows for convenient self-collection without the need for a medical professional inserting swabs into a patient's nasopharyngeal and/or oropharyngeal cavity. Moreover, saliva could be collected at home and have it sent to the laboratory in a safe box, hence omitting the need for the suspected person to travel to the diagnostic center and reduce the
transmission that could occur during the journey. This method could increase the scale of COVID-19 testing since specimen collection would require less manpower and time. Furthermore, direct to PCR from saliva specimens as a template for PCR detection reduces the cost of COVID-19 testing by two-folds: 1) exempting cost of trained professionals for specimen collection and the need of VTM and swab, and 2) removing the need for RNA Extraction process which are costly and time-consuming.

One of the highlights of our study is that we also conducted stability test for saliva specimen and found it was stable in cold (2-8°C) and room temperature in the laboratory (24-27°C) for up to 5 days. This finding is very important, since Indonesia is a vast country, and sending specimen from remote area to COVID-19 diagnostic laboratory could need few days. This situation might also be applicable to other countries with similar situation. Nevertheless, we would recommend that the saliva specimen should be transported in an icebox as outside temperature during the day could be hotter than 30°C[19]. Once arrived at the laboratory, the saliva specimen should also be processed as soon as possible. The sooner the diagnosis was made for the suspected person, the sooner subsequent measurement could be done, hence limiting the transmission of the disease.

Using saliva for direct-PCR for detection of COVID-19 especially potential for area where the diagnostic laboratory is located far from the suspected person and the capacity of diagnostic laboratory is overwhelmed. In area where diagnostic laboratory is close-by and have an adequate capacity, this method could be used as a subsequent screening in workplaces or schools after initial screening using NPOP specimen because frequent NPOP swab causes discomfort. The 90% positive agreement of this method is also superior compared to antigen swab [20,21]. The direct-PCR method is also an open system that could be implemented using other commercial RT-PCR for SARS-CoV-2 detection, although validation of their compatibility is recommended.
It is important to consider handling precautions during collection and processing of saliva via heat treatment alone since the method risks PCR inhibition from unpurified substances. This includes avoiding the use of powdered gloves during collection, transport and handling, where the powder entering the reactions are common causes of inhibited DNA amplification [22,23].

Good laboratory practices recommend the use of nitrile gloves in a molecular laboratory [24].

As seen in other reports, detection of SARS-CoV-2 infection can be lower in saliva when compared to NPOP [9–11]. This could arise from individuals bearing the virus only in their nasopharyngeal cavity but not in their oral cavity. NPOP swab collects specimen from both respiratory tract sites into a single tube of media, whereas saliva specimen only collects from the oral cavity. Further investigation on the viral pathway of infection is needed to understand these cases.

In this study, we did not conduct virus culture as definitive determination in the issue of disagreement of the NPOP and saliva specimens, because of the lack of airborne virus culture facility in our institution. Hence a future study on this would elucidate the cause of the non-concordance.

In conclusion, we validated the use of human saliva as a viable alternative specimen for the detection of SARS-CoV-2 via direct RT-qPCR. Saliva can be collected in a tube without additives and remained stable at cold and room temperature for five days of storage. Upon arrival in laboratory, saliva specimen can be treated against heat incubation method alone followed by direct addition as template for RT-qPCR reaction (Figure 4).

ACKNOWLEDGEMENTS
We would like to thank all the participating healthcare personnel and volunteers of our collaborating sites for their contribution in specimen collection: FIKK Atma Jaya, Rumah Sakit Atma Jaya, Rumah Sakit EMC Tangerang, Intibios Lab Mangga Besar, Intibios Lab Pantai Indah Kapuk, and PT Nalagenetik Riset Indonesia. We would also like to acknowledge all patients and respondents who have voluntarily provided their specimen for the purpose of this study, Sheila Jonnatan for her assistance in the early stage of the laboratory work, and members of PT Nalagenetik Riset Indonesia in assistance for QuickSpit™ design and development. This work was supported by COVID-19 Laboratory Center of Atma Jaya Catholic University of Indonesia (AJCUI).

AUTHOR CONTRIBUTIONS

Contributed to study design: CM, SA, MMMK, SV. Contributed to patient recruitment: SV, SSS, MMMK, ET, FC, KIJ. Performed laboratory experiments: SV, TAW, MMMK. Contributed to patient data management: SA, CM. Data analysis: CM, SV, MMMK, SA. Manuscript writing: CM, SV, SA, MMMK. Supervised and conceived the study: CM, SA, AI. All authors have reviewed the manuscript.

COMPETING INTERESTS

CM, SV, KIJ and AI are employees of Nalagenetics Pte Ltd, Singapore, holding the trademark of QuickSpit™.

REFERENCES

[1] Timeline of WHO’s response to COVID-19 [Internet]. [cited 2020 Oct 20]. Available from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/interactive-timeline#!
[2] Brian DA, Baric RS. Coronavirus genome structure and replication. Curr Top Microbiol Immunol. 2005;287:1–30.

[3] World Health Organization. Diagnostic testing for SARS-CoV-2 [Internet]. 2020. Available from: https://www.who.int/publications/i/item/diagnostic-testing-for-sars-cov-2.

[4] Wang W, Xu Y, Gao R, et al. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA [Internet]. 2020 [cited 2021 Mar 29];323:1843-1844. Available from: https://jamanetwork.com/journals/jama/fullarticle/2762997

[5] Zhang T, Cui X, Zhao X, et al. Detectable SARS-CoV-2 viral RNA in feces of three children during recovery period of COVID-19 pneumonia. J Med Virol [Internet]. 2020 [cited 2021 Mar 29];92:909–914. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jmv.25795.

[6] COVID-19 developments in Indonesia [Internet]. [cited 2021 Mar 31]. Available from: https://indonesien.ahk.de/infothek/news/news-details/covid-19-developments-in-indonesia.

[7] Hung KF, Sun YC, Chen BH, et al. New COVID-19 saliva-based test: How good is it compared with the current nasopharyngeal or throat swab test? J. Chinese Med. Assoc. Wolters Kluwer Health; 2020. p. 891–894.

[8] Wyllie AL, Fournier J, Casanovas-Massana A, et al. Saliva or Nasopharyngeal Swab Specimens for Detection of SARS-CoV-2. N Engl J Med. 2020;383:1283–1286.

[9] Xu R, Cui B, Duan X, et al. Saliva: potential diagnostic value and transmission of 2019-nCoV [Internet]. Int. J. Oral Sci. Springer Nature; 2020 [cited 2021 Mar 29]. p. 1–6. Available from: https://doi.org/10.1038/s41368-020-0080-z.
[10] Landry ML, Criscuolo J, Peaper DR. Challenges in use of saliva for detection of SARS CoV-2 RNA in symptomatic outpatients. J Clin Virol. 2020;130.

[11] Griesemer SB, van Slyke G, Ehrbar D, et al. Evaluation of specimen types and saliva stabilization solutions for SARS-CoV-2 testing. medRxiv. medRxiv; 2020.

[12] Alcoba-Florez J, González-Montelongo R, Íñigo-Campos A, et al. Fast SARS-CoV-2 detection by RT-qPCR in preheated nasopharyngeal swab samples. Int J Infect Dis. 2020;97:66–68.

[13] Smyrlaki I, Ekman M, Lentini A, et al. Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. Nat Commun [Internet]. 2020 [cited 2020 Oct 7];11:1–12. Available from: https://doi.org/10.1038/s41467-020-18611-5.

[14] Vogels CBF, Watkins AE, Harden CA, et al. SalivaDirect: A Simplified and Flexible Platform to Enhance SARS-CoV-2 Testing Capacity. Med. 2020;

[15] Iwasaki S, Fujisawa S, Nakakubo S, et al. Comparison of SARS-CoV-2 detection in nasopharyngeal swab and saliva. J. Infect. W.B. Saunders Ltd; 2020. p. e145–e147.

[16] Watkins AE, Fenichel EP, Weinberger DM, et al. Pooling saliva to increase SARS-CoV-2 testing capacity. medRxiv. medRxiv; 2020.

[17] Azzi L, Carcano G, Gianfagna F, et al. Saliva is a reliable tool to detect SARS-CoV-2. J Infect. 2020;81:e45–e50.

[18] Ott IM, Strine MS, Watkins AE, et al. Simply saliva: stability of SARS-CoV-2 detection negates the need for expensive collection devices. [cited 2020 Dec 10]; Available from: https://doi.org/10.1101/2020.08.03.20165233.

[19] WEATHER AND CLIMATE IN INDONESIA | Facts and Details [Internet]. [cited 2021
Mar 31]. Available from:
http://factsanddetails.com/indonesia/Nature_Science_Animals/sub6_8a/entry-4079.html.

[20] Mak GC, Cheng PK, Lau SS, et al. Evaluation of rapid antigen test for detection of SARS-CoV-2 virus. J Clin Virol. 2020;129:104500.

[21] Scohy A, Anantharajah A, Bodéus M, et al. Low performance of rapid antigen detection test as frontline testing for COVID-19 diagnosis. J Clin Virol. 2020;129:104455.

[22] Lomas J, Sunzeri F, Busch M. False-negative results by polymerase chain reaction due to contamination by glove powder. Transfusion [Internet]. 1992 [cited 2021 Mar 25];32:83–85. Available from: http://doi.wiley.com/10.1046/j.1537-2995.1992.32192116439.x.

[23] Broyles JM, O'Connell KP, Korniewicz DM. PCR-based method for detecting viral penetration of medical exam gloves. J Clin Microbiol [Internet]. 2002 [cited 2021 Mar 25];40:2725–2728. Available from: /pmc/articles/PMC120672/.

[24] Viana R V, Wallis CL. 3 Good Clinical Laboratory Practice (GCLP) for Molecular Based Tests Used in Diagnostic Laboratories [Internet]. [cited 2021 Mar 31]. Available from: http://users.ugent.be/~avierstr/.
FIGURE LEGENDS

Figure 1. Optimization of RNA-Extraction-free treatment. (A) Flowchart of eight different sample treatment prior SARS-CoV-2 detection (B) Bar graph represent means ± SEM of three SARS-CoV-2 positive specimens tested for each sample treatment. Asterisk * denotes the treatments for comparison, where 1 is the comparative method while 6 is the candidate method.

Figure 2. Linear regression of Ct values obtained from adding RNA extracted or heated-only saliva for RT-qPCR template. Data points in blue indicate Ct values for the detection of N-gene while orange for the detection of ORF1ab. Results obtained from saliva RNA template were plotted on x-axis, while heated-only saliva on y-axis. Line of best fit was plotted for both target genes to display direct proportion between the two variables.

Figure 3. Detection of SARS-CoV-2 target genes N-gene and ORF1ab remained stable for 5 days at cold and room temperature storage conditions. Relative Ct on y-axis plots the difference between Ct value obtained at a given day and Day 1 for the monitored sample. Data points in blue refer to samples stored at cold 4°C, while green refer to samples stored at room temperature. Straight lines display the average relative Ct obtained across storage days.

Figure 4. Schematic workflow for collection and treatment of human saliva specimen for RNA-extraction free detection of SARS-CoV-2 via RT-qPCR.
FIGURES

Figure 1
A

| Condition | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-----------|---|---|---|---|---|---|---|---|
| Specimen type | NPOP | Saliva |
| Sample treatment | 10ul proteinase K |
| | 37°C; 15’ |
| | 37°C; 30’ |
| | 95°C; 10’ |
| RNA detection | 13200 rpm; 2’ |
| | 13200 rpm; 2’ |
| | 13200 rpm; 5’ |

qRT-PCR for SARS-CoV 2

B

![Bar chart showing cycle threshold (Ct) for different treatments](chart)

- IC
- N-gene
- ORF1ab

* indicates statistical significance.
Figure 2

The figure shows a scatter plot with two lines of best fit. The equation for the blue line (FAM (N_gene)) is:

\[ y = 0.9999x + 0.7913 \]

with a coefficient of determination \( R^2 = 0.9291 \).

The equation for the orange line (VIC (ORF1ab)) is:

\[ y = 0.9659x + 2.4469 \]

with a coefficient of determination \( R^2 = 0.8367 \).
Figure 3
Figure 4

Saliva Specimen collection

Transported in RT to laboratory

Direct processing

NO

Kept at 2-8°C for maximum 5 days after collected

YES

2-10 second

seated for 1-10 minutes

30-100ul

95°C, 10 minutes

2-10 second

Kept at 2-8°C, for maximum 2 hours prior detection

RT-qPCR for SARS-CoV-2
TABLES

Table 1. SARS-CoV-2 detection from RNA extracted from NPOP versus saliva sample types.

| Saliva | NPOP |   |   | Total |
|--------|------|---|---|-------|
|        | Positive | Negative | Invalid |       |
| Positive | 60     | 0     | 2     | 62    |
| Negative | 11     | 32    | 2     | 45    |
| Invalid  | 7      | 1     | 1     | 9     |
| Total    | 78     | 33    | 5     | 116   |

Agreements % 95% CI
Overall 89.3 81.9 - 93.9
Positive 84.5 74.3 - 91.1
Negative 100 89.3 - 100
Table 2. Validation of heated-only saliva as RNA-extraction free treatment

| Heated Saliva | NPOP |     |     |     |
|--------------|------|-----|-----|-----|
|              | Positive | Negative | Invalid | Total |
| Positive     | 57     | 0    | 1    | 58   |
| Negative     | 15     | 31   | 3    | 49   |
| Invalid      | 9      | 1    | 1    | 11   |
| Total        | 81     | 32   | 5    | 118  |
| Agreements   | %      | 95% CI |     |     |
| Overall      | 85.4   | 77.4 - 91.0 |     |     |
| Positive     | 79.2   | 68.4 - 86.9 |     |     |
| Negative     | 100    | 89.0 - 100 |     |     |
Table 3. Performance of commercial kits using heated-only saliva as template for PCR.
Rates of valid, agreements and sensitivity were calculated as compared to results from the reference, Detection Kit for 2019 Novel Coronavirus (2019-nCoV) by Da An Gene Co.

| Commercial Kit | Sample size, n | Valid rate, % | Overall agreement, % | Estimated sensitivity, % |
|----------------|----------------|---------------|----------------------|--------------------------|
| Standard M nCov Real-Time Detection kit, SD Biosensor | 13 | 92.3 | 91.7 | 88.9 |
| Maccura SARS-CoV-2 Fluorescent PCR kit | 11 | 100 | 100 | 100 |
| Novel Coronavirus (COVID-19) Nucleic Acid Detection Kit (PCR-fluorescent probe), Ardent | 11 | 100 | 90.9 | 90.9 |
| MiRXES Fortitude Kit2.1 | 11 | 90.9 | 70 | 70 |
| Real-Q 2019 nCov Detection Kit, Biosewoom | 11 | 90.9 | 80 | 80 |
| 2019-Novel Coronavirus (2019-nCoV) Triplex RT-qPCR Detection Kit, Vazyme | 10 | 60 | 100 | 100 |
| 3S Non-direct SARS-CoV-2 RT-PCR Kit | 10 | 100 | 80 | 80 |
| Fosun COVID-19 RT-PCR Detection Kit | 12 | 91.7 | 100 | 100 |
Table 4. Implementation of QuickSpit™ as collection and treatment method of saliva specimen

| QuickSpit™    | NPOP     | Total |
|--------------|----------|-------|
|              | Positive | Negative | Invalid |     |
| Positive     | 34       | 0       | 0       | 34  |
| Negative     | 6        | 19      | 2       | 27  |
| Invalid      | 8        | 0       | 0       | 8   |
| Total        | 48       | 19      | 2       | 69  |

Agreements % 95% CI
Overall 89.8 79.5 - 95.3
Positive 85 70.9 - 92.9
Negative 100 83.2 - 100
SUPPLEMENTARY INFORMATION

S1. Specifications and characteristics of the commercial RT-qPCR kits, as derived from their respective manufacturer’s instructions for use

| Detection Kit for 2019 Novel Coronavirus (2019-nCoV), Da An Gene Co. | Target Genes-Reporter Dye | Internal Control-Reporter Dye | LOD (copies/ml) | Volume Reaction (µl) | Volume Template (µl) | No. of Cycles | Cycle Cut-off |
|---|---|---|---|---|---|---|---|
| | N gene-FAM | ORF1ab-VIC | RNase P-Cy5 | 500 | 25 | 5 | 45 | 40 |
| Standard MnCov Real-Time Detection kit, SD Biosensor | ORF1ab (RdRP)-FAM | ORF1ab (RdRP)-FAM | Internal control A (Pseudovirus)-Cy5 | 250 | 20 | 10 | 40 | 36 |
| Maccura SARS-CoV-2 Fluorescent PCR kit | ORF1ab-FAM | E gene-VIC/HEX | N gene-Cy5 | 1000 | 20 | 20 | 40 | 38 |
| Novel Coronavirus (COVID-19) Nucleic Acid Detection Kit (PCR-fluorescent probe), Ardent | ORF1ab (RdRp)-FAM | ORF1ab (RdRp)-FAM | N gene-VIC | 400 | 15 | 5 | 45 | 40 |
| MiRXES Fortitude kit 2.1 | ORF1ab region 1-FAM | ORF1ab region 2-HEX | Synthetic DNA oligo-Cy5 | 200 | 20 | 5 | 42 | 40 |
| Real-Q 2019 nCov Detection Kit, Biosewoom | RdRP gene-FAM | E gene-HEX/VIC | HRP-Cy5 | 3125 | 20 | 5 | 40 | 38 |
| Kit Description                                      | Assay Components                                      | Temp (°C) | Time (min) | Incubation (min) | Fluorescent Dyes |
|------------------------------------------------------|--------------------------------------------------------|-----------|------------|------------------|------------------|
| 2019-Novel Coronavirus (2019-nCoV) Triplex RT-qPCR Detection Kit, Vazyme | ORF1ab-FAM, N gene-ROX/Texas Red, RNase P-Cy5         | 200       | 30         | 20               | 45               | 38               |
| 3S Non-direct SARS-CoV-2 RT-PCR Kit                  | N gene-FAM, F1ab-HEX, ⫿-actin-VIC/HEX                 | 300       | 15         | 10               | 45               | 40               |
| Fosun COVID-19 RT-PCR Detection Kit                  | ORF1ab-FAM, E gene-ROX, N gene-JOE, Lentivirus-Cy5   | 300       | 20         | 10               | 40               | 36               |
S2. Comparison of eight different sample treatments prior to SAR-CoV-2 detection via PCR for negative specimens. Bar graph represent means ± SEM for two SARS-CoV-2 negative specimens tested for each sample treatment illustrated in Figure 1A.
S3. Detection of SARS-CoV-2 target genes N and ORF1ab plotted as the Ct value obtained for monitored sample. Data points collected in Day 1 are displayed as white circles with black outline. Blue points refer to samples stored at cold while green at room temperature. Straight lines drawn monitor changes for samples starting with the highest and lowest Ct value in Day 1.
S4. Responses from user research of QuickSpit™ prototyping. (A) Demographics of respondents. (B) Frequency of features referenced during the survey.

A

![Demographics chart]

B

| Feature          | Frequency of Responses, % |
|------------------|---------------------------|
| Instructions     | Strongly agree easy to follow |
|                  | Prefer video instructions |
|                  | Prefer hardcopy text instructions |
| Mouthpiece       | Strongly against for straw |
|                  | Against triangle-shaped |
|                  | Strong preference for round-shaped |
|                  | Easy to open and close the screw-cap |
| Hold             | Necessary for both hands to hold onto device |
|                  | Size of vial convenient to hold |
| Volume           | Unclear indication line on vial for 1.5mL |
| Hygiene          | Mouthpiece disposed into a secondary bag |
|                  | Wipe the lip of vial with tissue |

CC-BY-ND 4.0 International license

which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-ND 4.0 International license.