Supporting Information

On-Site Viral Inactivation and RNA Preservation of Gargle and Saliva Samples Combined with Direct Analysis of SARS-CoV-2 RNA on Magnetic Beads

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| Study            | Sample | Inactivation/Extraction                                                                 | RNA Release & Stability                  | Detection Method                  | Sensitivity                     |
|------------------|--------|----------------------------------------------------------------------------------------|------------------------------------------|-----------------------------------|---------------------------------|
| VIP-Mag Method   | BOTH   | ✓ Single Tube Method (sample collection, inactivation, lysis, & maintains RNA stability) | ✓ 1 week at 4°C & Room Temperature      | RT-qPCR                          | 25 RNA copies/200 µL of sample  |
|                  |        | Heat at 55°C, 10 min                                                                   | Directly inputted into RT-qPCR without the need for elution/purification |                                   |                                 |
| Ranoa et al., 2020 | Saliva | ✓ Heat at 95°C for 30 min                                                               | 24 hours at 4°C (RNA)                    | RT-qPCR                          | 500–1000 viral particles/mL of saliva |
|                  |        | Heat at 95°C for 30 min TBE buffer and Tween 20                                         | Directly inputted into RT-qPCR without the need for elution/purification |                                   |                                 |
| Vogels et al., 2020 | Saliva | ✓ Heat at 95°C for 5 min Proteinase K                                                    | 1 week at 30°C, 4°C & Room Temperature (SARS-CoV-2 in saliva pre-inactivation/extraction) | RT-qPCR                          | 6–12 SARS-CoV-2 copies/µL of saliva |
|                  |        | Proteinase K                                                                            | Directly inputted into RT-qPCR without the need for elution/purification |                                   |                                 |
| Lalli et al., 2021 | Saliva | ✓ Heat at 65 °C for 15 min, 95 °C for 5 min, and cooled to 4 °C                            | RNA stability not mentioned              | RT-qPCR                          | 100 viral genomes/reaction       |
|                  |        | Heat & RNAsecure & Proteinase K to each sample                                          | Directly inputted into RT-qPCR and LAMP without the need for elution/purification |                                   |                                 |
| Yang et al., 2021 | Saliva | ✓ Heat at 95 °C for 10 min Saliva stabilization solution (5 mM TCEP, 2 mM EDTA, 29 mM NaOH, 100 µg/mL Proteinase K) | 4 days at 4°C (RNA)                     | LAMP                             | 200 virions/µL of saliva         |
|                  |        |                                                                                       |                                          |                                   |                                 |
| Gargle           | ✓      |                                                                                       |                                          |                                   |                                 |
| Tilley et al., 2021 | Saline solution | Heat at 65 °C for 20 min and cool to room temperature for 5 min | RNA stability not mentioned | RT-qPCR Single-tube hemi-nested real-time-qPCR (STHN-RT-qPCR) to enhance the overall sensitivity | 97% match to NPS positive samples (viral number or copy number not mentioned) |
|------------------|-----------------|---------------------------------------------------------------|-----------------------------|------------------------------------------------------------|---------------------------------------------------------------------|

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Table S2. A summary of studies on the detection of SARS-CoV-2 in saliva. Inactivation/extraction method, RNA stability, detection method, and sensitivity comparison with nasopharyngeal swabs (NPS) analysis are summarized. The checkmarks indicate that the study addressed a certain aspect.

| Study                | Sample | Inactivation/Extraction | RNA Stability | Detection Method | Comparison to NPS positive samples |
|----------------------|--------|-------------------------|---------------|-----------------|-----------------------------------|
| Altawalah et al., 2020 | Saliva | ✓                       | X             | ✓               | ✓                                 |
|                      | Mixed in VTM | MagMax Viral/Pathogen Nucleic Acid Isolation Kit | Not mentioned | RT-qPCR         | 91% match to NPS                  |
| Aita et al., 2020    | Saliva | X                       | X             | ✓               | ✓                                 |
|                      | Salivette® | Not mentioned | Not mentioned | RT-qPCR Digital drop PCR | 100% match to NPS positive samples (One sample was positive for saliva, but not for NPS.) |
| Azzi et al., 2020    | Saliva | ✓                       | X             | ✓               | ✓                                 |
|                      | PBS dilution | QIAmp Viral RNA mini kit | Not mentioned | RT-qPCR         | 100% match to NPS                  |
| Byrne et al., 2020   | Saliva | ✓                       | X             | ✓               | ✓                                 |
|                      | Saliva | QIAmp Viral RNA Mini Kit | Not mentioned | RT-qPCR         | 86% match to NPS                  |
| Chen et al., 2020    | Saliva | ✓                       | X             | ✓               | ✓                                 |
|                      | Mixed in VTM | Xpert Xpress SARS-CoV-2 assay | Not mentioned | RT-qPCR Xpert Xpress SARS-CoV-2 assay | 84% positive in both NPS and saliva, 10% positive in NPS only, and 5.2% positive in saliva only |
| Cheuk et al., 2020   | Saliva | ✓                       | X             | ✓               | ✓                                 |
|                      | MagNA Pure LC 2.0 | Not mentioned | RT-qPCR | 85% match to NPS |
| Güçlü et al., 2020   | Saliva | ✓                       | X             | ✓               | ✓                                 |
|                      | EZ1 Virus Kit Qiangen | Not mentioned | RT-qPCR | 85% match to NPS |
| Han et al., 2020     | Saliva | ✓                       | ✓             | ✓               | ✓                                 |
|                      | Seegene platform | Positivity decreased to 33% and 11%, at week 2 and 3, respectively. | RT-qPCR | 80% match to NPS |
| Hanson et al., 2020  | Saliva | ✓                       | ✓             | ✓               | ✓                                 |
|                      | Diluted in ARUP laboratories | Hologic Apteima Panther platform | 5 days at 4°C & Room Temperature | RT-qPCR | 94% match to NPS |
| Study              | Transport Medium                  | Saliva | Mixed with PBS | DNA/RNA Shield™ solution | Not mentioned | RT-qPCR | Match to NPS |
|--------------------|----------------------------------|--------|----------------|--------------------------|---------------|---------|--------------|
| Iwasaki et al., 2020 | Saliva                           | ✓      |                | QIAamp Viral RNA Mini Kit | Not mentioned | RT-qPCR | 100% match to NPS |
| Kojima et al., 2020 | Saliva                           | ✓      |                | RNA purification kit, Norgen Biotek Corp | Not mentioned | RT-qPCR | Physician supervised: 90% match to NPS Self collected: 66% match to NPS |
| Leung et al., 2020  | Saliva                           | ✓      |                | MagMAX viral RNA isolation kit | Not mentioned | RT-qPCR | 79% match to NPS |
| Mao et al., 2020    | Saliva                           | ✗      |                | Not mentioned | Not mentioned | RT-qPCR | Saliva alone had a 74% match to NPS, but if there was sputum, then match increased to 93% |
| McCormick-Baw et al., 2020 | Saliva                           | ✓      |                | Cepheid Xpert Xpress SARS-CoV-2 assay | Not mentioned | RT-qPCR | 96% match to NPS |
| Migueres et al., 2020 | Saliva                           | ✓      |                | Hologic Aptima Panther platform | Not mentioned | RT-qPCR | 88% and 95% match for asymptomatic and symptomatic patients, respectively |
| Pasomsb et al., 2020 | Saliva                           | ✓      |                | bioMerieux lysis buffer | Not mentioned | RT-qPCR | 98% match to NPS |
| Rao et al., 2020    | Saliva                           | ✓      |                | MagNA Pure 96 DNA and Viral NA Small Volume extraction kit | Not mentioned | RT-qPCR | SARS-CoV-2 was detected more frequently using saliva (93%) than NPS (52%) |
| Senok et al., 2020  | Saliva                           | ✓      |                | Chemagic™ 360 Nucleic Acid Extractor | Not mentioned | Rt-qPCR | 73% match to NPS |
| To et al., 2020     | Saliva                           | ✓      |                | NucliSENS easyMAG | Not mentioned | RT-qPCR | 92% match to NPS |
| Uwamino et al., 2020 | Saliva                           | ✗      |                | Room temperature for 7 days | Not mentioned | RT-qPCR | 32 positive by both NPS and saliva, 15 by NPS only, 11 by saliva only |
| Study                  | Specimen | Initial    | Lysis      | Detection Method | Match to NPS |
|-----------------------|----------|------------|------------|-----------------|--------------|
| Wong et al., 2020     | Saliva   | ✓          | ✗          | RT-qPCR         | 85% match to NPS |
|                       |          | MagNA Pure LC 2.0 | Not mentioned |                 |              |
|                       |          | MagNA Pure 96 |            |                 |              |
| Wyllie et al., 2020   | Saliva   | ✓          | ✓          | RT-qPCR         | 81% were positive by saliva and 71% by NPS |
|                       |          | MagMAX     |            |                 |              |
|                       |          | Viral/Pathogen Nucleic Acid Isolation kit | Mentioned stable for 25 days at Room Temperature, but not tested | | |
| Vaz et al., 2020      | Saliva   | ✓          | ✗          | RT-qPCR         | 96% match to NPS |
|                       |          | Diluted in PBS | Not mentioned |                 |              |
|                       |          | QIAGEN QIAamp® RNA Mini Kit | | | |
| Yokota et al., 2021   | Saliva   | ✓          | ✗          | RT-qPCR         | 86% sensitivity for NPS and 92% for saliva |
|                       |          | Diluted in PBS | Not mentioned |                 |              |
|                       |          | QIAasympohy DSP Virus/Pathogen kit and QIAamp Viral RNA Mini Kit | | | |

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Table S3. A summary of studies on the detection of SARS-CoV-2 in gargle. Inactivation/extraction method, RNA stability, detection method, and sensitivity comparison to nasopharyngeal swabs (NPS) analysis are summarized. The checkmarks indicate that the study addressed certain aspects.

| Study                  | Sample                  | Inactivation/Extraction | RNA Stability | Detection Method       | Sensitivity/Comparison to NPS positive samples |
|------------------------|-------------------------|-------------------------|---------------|------------------------|-----------------------------------------------|
| Goldfarb et al., 2021  | Gargle                  | ✓                       | ✓             | RT-qPCR                | 98% sensitivity, 39/40 NPS confirmed patients tested positive using gargle |
|                        | Saline solution         |                         |               |                        |                                               |
|                        | QiaSymphony automated extractor using the DSP virus/pathogen minikit |                         |               |                        |                                               |
|                        | Cepheid Xpert Xpress SARS-CoV-2 assay |                         |               |                        |                                               |
|                        | 2 days at Room Temperature |                         |               |                        |                                               |
| Kandel et al., 2021    | Gargle                  | ✓                       |✗             | RT-qPCR                | 90% match to NPS                              |
|                        | Saline solution         |                         |               |                        |                                               |
|                        | Heated at 56°C for 30 mins in a dry bath filled with thermal beads and vortexed for 30 secs. |                         |               |                        |                                               |
|                        | TNA lysis buffer (plus carrier RNA, and MS2 phage internal control) |                         |               |                        |                                               |
|                        | MagBind Viral RNA Xpress kit |                         |               |                        |                                               |
| Lopez-Lopes et al., 2020 | Gargle                 | ✓                       |✗             | RT-qPCR                | Not all samples had a paired NPS, but study generally found that Ct values of throat washes were comparable to NPS but higher |
|                        | Saline solution         |                         |               |                        |                                               |
|                        | Automated extraction (Bio Gene, Quibasa or Abbott M2000) |                         |               |                        |                                               |
| Malecki et al., 2021   | Gargle                  | ✓                       |✗             | RT-qPCR                | Screened 924 healthcare workers, 26 were positive |
|                        | Saline solution         |                         |               |                        |                                               |
|                        | Not mentioned           |                         |               |                        |                                               |
| Paré et al., 2021      | Gargle                  | ✓                       |✗             | In house laboratory developed (LD) NAAT | 1297 adult samples processed. Overall sensitivity was 98% for NPS and 90% for gargles. |
|                        | Natural spring water    |                         |               |                        |                                               |
| Poukka et al., 2021 | Both | ✓ | ✓ | ✓ | ✓ |
|--------------------|------|---|---|---|---|
| Saliva & Gargle    | Viscous samples were diluted with PBS and vortexed | Not mentioned | One step RT-qPCR | Saliva had 100% sensitivity |
|                    | Chemagic Viral300 DNA/RNA kit H96 | | | Gargle had 97% sensitivity |

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Table S4. A summary of reported methods that were used to detect SARS-CoV-2 in pooled samples. The checkmarks indicate that the study addressed certain aspects.

| Study            | Sample    | Inactivation/Extraction | RNA Stability          | Detection Method     | Results                                                                 |
|------------------|-----------|-------------------------|------------------------|----------------------|-------------------------------------------------------------------------|
| VIP-Mag Method   | Both      | ✓                       | ✓                      | ✓                    | Positive detectable even after diluting by 32 times                     |
| Saliva           |           | Single Tube Method (sample collection, inactivation, lysis, & maintains RNA stability) Heat at 55°C, 10 min | 1 week at 4°C & Room Temperature (RNA) | RT-qPCR               |                                                                          |
| Barat et al., 2021 | Saliva    | Proteinase K, vortexed and heated for 5 min at 95°C NucliSENS easyMAG Panther Fusion Cobas 6800 | Not mentioned | RT-qPCR               | 90-94% sensitivity in 5 pooled samples                                |
| Bokelmann et al., 2021 | Gargle | Lysis/binding buffer (Tris-HCl, LiCl, LiDS, EDTA, DTT) Quick extract (Lucigen) | Not mentioned | RT-qPCR capture and improved loop-mediated isothermal amplification (CAP-LAMP) | 1 positive in 25 pooled samples can be detected                          |
| Kellner et al., 2021 | Gargle | Guanidine thiocyanate KingFisher magnetic particle processor DNaseI for 15 mins at 37°C QuickExtract DNA extraction solution (Lucigen) | Not mentioned | RT-qPCR LAMP           | 1 positive in 100 pooled samples can be detected                        |
| Willeit et al., 2021 | Gargle | 10 pooled samples mixed using KingFisher Flex mixer Lysis buffer (Tris, GITC, EDTA, 2% Triton) | Not mentioned | RT-qPCR               | This method was used to screen 10 734 participants from 245 schools in Austria. |
2 M 1,4-dithiothreitol added to reduce viscosity

X-100, DTT) added and incubated for 10 min at room temperature

KingFisher Flex Magnetic Particle Processor System

Carboxylated magnetic bead CyBio Felix System

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Comparison of Tap Water and Saline Used for Collecting Gargle Samples

To explore whether tap water is suitable for collecting a gargle sample, we compared the stability of SARS-CoV-2 viral RNA in tap water gargle and saline gargle samples. We obtained pooled tap water gargle and pooled saline gargle from SARS-CoV-2 negative volunteers. We added 65, 390, or 3900 copies of viral RNA to each type of the pooled gargle samples. We analyzed these samples after they were stored at room temperature for 2 h. The results show that the tap water gargles containing 65 or 390 copies of the viral RNA required higher threshold cycles (Ct) to achieve detection than those for their saline gargle counterparts (Figure S1A). We also tested three saline gargle and three tap water gargle samples collected from a SARS-CoV-2 positive patient (Figure S1B). The Ct values are consistently higher for the tap water gargle samples than for the saline gargles samples. These results indicate lower concentrations of the viral RNA in tap water gargle than in saline gargle samples, probably because of more degradation of the viral RNA in tap water. Saline was used for the subsequent collection of all the gargle samples in this study.

Additional Information on Developing a Viral Inactivation and RNA Preservation (VIP) Buffer.

We first tested commercially available QuickExtract plant DNA extract solution (containing SDS) and RLT lysis buffer (containing guanidinium isothiocyanate; QIAgen), as well as the addition of 2-mercaptoethanol (2-ME), proteinase K, and Triton X-100 (Figure S2). The analyses of gargle samples containing 3900 copies of viral RNA show that a lower threshold cycle (Ct) was obtained for the detection of the viral RNA when the RLT buffer was used as compared to the QuickExtract plant DNA extract solution (Figure S2). We chose to use the RLT buffer with the addition of 2-mercaptoethanol (2-ME), proteinase K, and Triton X-100. Proteinase K and 2-mercaptoethanol were used to denature proteins and digest RNase enzymes that would otherwise degrade RNA. Chellappan et al. (J. Ind. Microbiol. Biotechnol. 2011, 38(6), 743–752. doi:10.1007/s10295-010-0914-3) showed that 1% 2-ME enhanced the activity of proteinase K although 5% 2-ME reduced its activity. Triton X-100 was used to enhance inactivation of SARS-CoV-2 by destroying envelopes of virions. The optimum
concentrations of these reagents in the VIP buffer were 1% 2-ME, 2.5% Triton X-100, and 170 ng/μL proteinase K.

Additional Information on Enhancing the Recovery of Low Amounts of RNA.

We compared the use of a commercially available RNA carrier and glycogen for enhancing the recovery of low amounts of RNA from gargle samples. We added 17, 34, or 68 ng/μL of glycogen or 1.7 ng/μL of Carrier RNA (1 μg per sample, recommended by MagMAX viral RNA isolation kit) in VIP buffer. We mixed 600 μL of VIP buffer with 200 μL of gargle samples containing 390 or 3900 copies of viral RNA. As shown in Figure S3, the addition of glycogen reduced the threshold cycles (Ct) needed for the detection of 390 copies of viral RNA, suggesting a better recovery of the viral RNA for detection. The effect of “Carrier RNA” and glycogen on the higher concentration (3900 copies) of viral RNA is minimum. To achieve efficient recovery of minute amounts of viral RNA from the samples, we added glycogen into VIP buffer to a final concentration of 17 ng/μL.
Figure S1. Detection of SARS-CoV-2 RNA in tap water gargle and saline gargle samples. (A) Viral RNA were added to SARS-CoV-2 negative tap water gargle or saline gargle samples and detected after storage at room temperature for 2 h. (B) Detection of SARS-CoV-2 RNA in three tap water gargle and three saline gargle samples collected from a SARS-CoV-2 positive patient. The error bars represent one standard deviation of triplicate measurements. NTC (no template control) is negative control. ND indicates no detectable SARS-CoV-2 RNA.
Figure S2. Detection of SARS-CoV-2 RNA in gargle samples treated with different reagents. Quick Plant DNA EX stands for Quick Plant DNA Extract. RLT stands for RLT lysis buffer. PK indicates proteinase K, and TX indicates Triton X-100. The error bars represent one standard deviation of triplicate measurements. NTC (no template control) is negative control. ND indicates no detectable SARS-CoV-2 RNA.
Figure S3. Detection of SARS-CoV-2 RNA in gargle samples treated with glycogen or carrier RNA. The gargle samples contained either 390 or 3900 copies of SARS-CoV-2 RNA. The error bars represent one standard deviation of triplicate measurements. NTC (no template control) is negative control. ND indicates no detectable SARS-CoV-2 RNA.
Figure S4. Detection of SARS-CoV-2 RNA in saliva and gargoyle samples treated with either freshly prepared VIP buffer or the VIP buffer stored at room temperature for six months. The saliva and gargoyle samples each contained 390 copies of viral RNA. The error bars represent one standard deviation of triplicate measurements. NTC stands for no template control. ND indicates no detectable SARS-CoV-2 RNA.
Figure S5. Detection of SARS-CoV-2 RNA in the absence or the presence of commercial beads. Solid Phase Reversible Immobilization select beads (SPRIselect beads) and silica-based beads (TurboBeads) were tested. Ten or 20 μL of SPRIselect or TurboBeads were washed three times with RNase-free water and then added into a sample of 2000 copies of SARS-CoV-2 RNA. The samples were analyzed using RT-qPCR. The error bars represent one standard deviation of triplicate measurements. NTC denotes no template control. ND indicates no detectable SARS-CoV-2 RNA. PC indicates positive control. Similar Ct values from the reactions containing SARS-CoV-2 RNA with or without the presence of SPRIselect beads indicate that the SPRIselect beads did not affect the RT-qPCR detection.
Figure S6. Comparison of different concentrations of PEG 8000 in the beads-binding buffer for concentrating SARS-CoV-2 RNA. The error bars represent one standard deviation of triplicate measurements. NTC (no template control) is negative control containing all reagents including 36% PEG. ND indicates no detectable SARS-CoV-2 RNA.
Figure S7. Detection of 65, 390, or 3900 copies of SARS-CoV-2 RNA in saliva and gargle samples using the VIP-Meg-RT-qPCR method. The error bars represent one standard deviation of triplicate measurements. NTC (no template control) is negative control. ND indicates no detectable SARS-CoV-2 RNA.
Figure S8. Recovery of viral RNA from RNase-free water and pooled SARS-CoV-2 negative gargle samples. RNase-free water and pooled gargle samples from healthy volunteers were each spiked with 65, 390, or 3900 copies of SARS-CoV-2 RNA. The samples were analyzed using the VIP-Meg-RT-qPCR method. The error bars represent one standard deviation of triplicate measurements. NTC (no template control) is negative control. ND indicates no detectable SARS-CoV-2 RNA.
Figure S9. Standard curve from the RT-qPCR analysis of the N1 gene segment (CDC). The log values of the numbers of pure SARS-CoV-2 RNA are plotted against the corresponding Ct values. E represents PCR efficiency which was calculated using the equation: $E = -1 + 10(-1/slope)$, where slope refers to the slope of the standard curve. This standard curve was used to quantify the amounts of SARS-CoV-2 RNA in samples.
Figure S10. SARS-CoV-2 RNA levels in saliva samples collected four times a day on five days. The samples were collected from the first SARS-CoV-2 positive patient volunteer from the sixth to the tenth day. Lines represent the mean of duplicates, shown individually as symbols. NTC (no template control) is negative control.
Figure S11. SARS-CoV-2 RNA levels in gargle samples collected four times a day on seven days. The gargle samples were collected from the second SARS-CoV-2 positive patient volunteer from the fourth to the 11th day. Lines represent the mean of duplicates, shown individually as symbols. NTC (no template control) denotes negative control.