PLOS ONE

Rapid processing of SARS-CoV-2 containing specimens for direct RT-PCR

Piotr Chomczynski[1]*, Peter W. Chomczynski[1], Amy Kennedy[1], Michal Rymaszewski[1], William W. Wilfinger[1], Judith A. Heiny[2], Karol Mackey[1]

1 Molecular Research Center, Inc., Cincinnati, Ohio, United States of America, 2 Department of Pharmacology & Systems Physiology, University of Cincinnati, Cincinnati, Ohio, United States of America

* piotr@mrcgene.com

Abstract

Widespread diagnostic testing is needed to reduce transmission of COVID-19 and manage the pandemic. Effective mass screening requires robust and sensitive tests that reliably detect the SARS-CoV-2 virus, including asymptomatic and pre-symptomatic infections with a low viral count. Currently, the most accurate tests are based on detection of viral RNA by RT-PCR. We developed a method to process COVID-19 specimens that simplifies and increases the sensitivity of viral RNA detection by direct RT-qPCR, performed without RNA purification. In the method, termed Alkaline-Glycol Processing (AG Processing), a SARS-CoV-2-containing biological specimen, such as saliva or a swab-collected suspension, is processed at pH 12.2 to 12.8 for 5 min at room temperature. An aliquot of the AG-processed specimen is used for detection of SARS-CoV-2 RNA by direct RT-qPCR. AG processing effectively lyses viruses and reduces the effect of inhibitors of RT-PCR that are present in biological specimens. The sensitivity of detecting viral RNA using AG processing is on par with methods that include a viral RNA purification step. One copy of SARS-CoV-2 virus per reaction, equivalent to 300 copies per ml of saliva, is detectable in the AG-processed saliva. The LOD is 300 viral copies per ml of initial saliva specimen. AG processing works with saliva specimens or swab specimens collected into Universal Transport Medium, is compatible with heat treatment of saliva, and was confirmed to work with a range of CDC-approved RT-qPCR products and kits. Detection of SARS-CoV-2 RNA using AG processing with direct RT-qPCR provides a reliable and scalable diagnostic test for COVID-19 that can be integrated into a range of workflows, including automated settings.

Introduction

The COVID-19 pandemic has created a great demand for molecular tests that detect viral infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the RNA virus responsible for coronavirus disease 2019 (COVID-19). Currently, the most accurate diagnostic tests for COVID-19 are based on detection of SARS-CoV-2 RNA. However, virus-containing biological specimens collected from patients contain components that negatively affect

detection of viral RNA by degrading RNA and inhibiting RT-PCR. Several strategies have been developed to mitigate the negative actions of these components.

Commonly used tests to detect viral RNA involve removal of extraneous components from a biological specimen by RNA purification. These tests include two steps: 1) a purification step to extract viral RNA from a patient sample (nasopharyngeal swab, sputum, or other); followed by 2) a quantitative reverse transcription PCR (RT-qPCR) assay to detect SARS-CoV-2 RNA. Effective target sequences for detecting SARS-CoV-2 RNA by RT-qPCR are located in the nucleocapsid (N) gene or the spike (S) protein gene of SARS-CoV-2, and the most effective target is the N1 sequence of the N gene [1]. The lower limit of detection (LOD) of RT-qPCR performed on samples of purified SARS-CoV-2 RNA using N1 primers is approximately 10 copies of viral RNA per reaction, with a cycle threshold (C_T) of 36 [2]. For automated detection of SARS-CoV-2 using cartridges for viral RNA isolation followed by RT-PCR, the LOD for detecting viral RNA was 14.8 copies per reaction and 365 copies per ml of a viral specimen, using primers for the S gene [3]. Alternatively, RT and a Loop-Mediated Isothermal Amplification (LAMP) method can be used to detect SARS-CoV-2 RNA. The detection sensitivity of an RT-LAMP test for SARS-CoV-2 was 100 copies of purified viral RNA per reaction [4].

Other RT-qPCR tests have been developed that eliminate the viral RNA purification step, in which a minimally processed virus-containing specimen is added directly to the RT-qPCR assay (direct RT-qPCR). Direct RT-qPCR offers advantages for streamlining testing because it is faster and simpler to implement than tests which require an initial RNA purification step. Most direct RT-PCR tests use saliva because it is more sensitive than nasopharyngeal swabs for detection of SARS-CoV-2 and it is simpler and safer to collect [5].

Several direct RT-qPCR diagnostic tests have been developed in which heat-processed saliva samples or viral suspensions in Universal Transport Medium (UTM) are diluted in a buffer, and an aliquot of the treated sample is added directly to a reagent mix to perform RT-PCR, without RNA purification. In one test, a viral suspension is heated at 95C for 10 min, then used for direct RT-qPCR [6]. For a low viral load ($C_T > 30$); this method was not satisfactory and test results required further confirmation. In other direct RT-PCR tests, saliva was diluted with a buffer and heated at 95 C for 30 min and then supplemented with Tween-20 [7] or heated at 65 C for 10 min [8]; an aliquot of the heat-treated, diluted sample was used for direct RT-PCR. The LOD for these tests, evaluated using saliva spiked with γ-irradiated SARS-CoV-2, was 1,000 copies of SARS-CoV-2 virus per ml of saliva [7], or 6,600 per ml of a viral suspension spiked with synthetic SARS-CoV-2 RNA [8]. Another direct RT-PCR test for detecting SARS-CoV-2 RNA used saliva treated with dithiothreitol (Sputasol) and a ribonuclease inhibitor [9]. For this method, the LOD of the final RT-PCR mix, evaluated using saliva spiked with a viral RNA template of 99 bp, was 12 copies of RNA template per reaction (equivalent to 14,000 copies of viral RNA template per ml of saliva). Another publication described a method in which saliva samples were treated with proteinase K and then heated before being used in a direct RT-PCR assay [10]. The reported LOD for detecting SARS-CoV-2, evaluated using SARS-CoV-2 positive saliva, was 6,000 virus copies per ml of saliva. In direct RT-PCR tests, addition of non-ionic detergents, such as Tween-20 and Triton X-100, to saliva or swab-derived samples was shown to increase the sensitivity of SARS-CoV-2 RNA detection [7, 11].

Our goal was to further simplify detection of SARS-CoV-2 RNA by direct RT-PCR and to enhance its sensitivity. We focused on the initial processing of clinical specimens because this step is time-consuming and critically affects the sensitivity of subsequent RNA detection assays.

We developed a method, termed Alkaline-Glycol Processing (AG processing), patent pending [12], in which a virus-containing specimen is briefly incubated in an alkaline-glycol solution and the AG-processed specimen is assayed for the presence of a viral RNA by direct RT-

qPCR. The method builds on a previously described direct PCR assay for detecting DNA in biological samples [13, 14]. It was found that concentrated polyglycols in alkaline aqueous solution act as chaotropes, which increase the alkalinity of a lysis solution and promote alkaline lysis of biological samples.

We tested detection of viral RNA using AG processing with human saliva specimens from multiple donors and virus-containing specimens suspended in Universal Transport Media (UTM) or Hanks' medium. Direct RT-qPCR assays were performed with a range of RT-qPCR products and kits, using γ-irradiated or heat-inactivated virus particles as template. We determined the LOD for detecting viral RNA using saliva spiked with a defined quantity of γ-irradiated or heat-inactivated SARS-CoV-2 virus particles or a defined quantity of synthetic SARS-CoV-2 viral RNA. We report that AG processing used with direct RT-qPCR provides a highly accurate and sensitive diagnostic test for COVID-19. The method is economical, scalable, and can be integrated into a range of diagnostic workflows and automated settings.

Materials and methods

RT-qPCR

RT-qPCR tests were performed using commercially-available one-step RT-qPCR kits. The following kits were used: TaqPath™ 1-Step RT-qPCR Master Mix, CG (Thermo Fisher Scientific, Waltham, MA, USA; cat. no. A15300). qScript™ XLT 1-Step RT-qPCR ToughMix™ ROX™ (Quantabio, Beverly, MA, USA; cat. no. 95133–500). GoTaq® Probe 1-Step RT-qPCR System (Promega Corporation, Madison WI, USA; cat. no A6121). KiCqStart® Probe qPCR Ready-Mix™, ROX™ (MilliporeSigma, St. Louis, MO, USA; cat. no. KCQS06). The RT-qPCR reaction parameters followed each manufacturer's recommendations.

Reference materials

The following materials were used as substitutes for SARS-CoV-2 live virus: NIAID, NIH: SARS-Related Coronavirus 2, Isolate USA-WA1/2020, Heat inactivated (NR-52286; 1.16×10^9 genome equivalents per ml, BEI Resources, Manassas, VA, USA); γ-irradiated SARS-CoV-2 (NR-52287, 1.7×10^9 genome equivalents per ml, BEI Resources, Manassas, VA, USA); synthetic SARS-CoV-2 RNA, 99.9% genome coverage, Control 1 (MT007544.1) and Control 2 (MN 908947.3) (Twist Bioscience, San Francisco, CA, USA). Positive control plasmid contained SARS-CoV-2 nucleocapsid gene cDNA at 2×10^5 copies/μl (2019-nCoV_N Positive Control Plasmid, Integrated DNA Technologies, Coralville, IA, USA. Cat no. 10006625). Primers and detection probes for N1 and N2 regions of the nucleocapsid gene were sourced from Integrated DNA Technologies (cat. no. 10006770) that were manufactured using the U.S. CDC sequences and QC qualified under a U.S. CDC Emergency Use Authorization. The human RPP30 gene was used to confirm amplification of a gene known to be expressed in saliva (RPP30 primer set, exon 1–2, Integrated DNA Technologies, Coralville, IA, USA, Cat. no. Hs.PT.58.19785851).

Human saliva specimens

Human saliva samples (sputum) from individual donors were collected by Molecular Research Center, Inc, in accordance with an approved IRB protocol (MRC C19 protocol, "Collection of saliva for detection of viral ribonucleic acid (RNA)", IntegReview IRB, Austin, TX.). All participants were adults and gave written informed consent prior to study inclusion.

Alkaline-glycol processing of saliva

Saliva was spiked with known quantities of a SARS-CoV-2 reference material. AG processing was performed by mixing one volume of an aqueous solution containing 65% (v/v) polyethylene glycol 200 (PEG200, CAS 25322-68-3) and 45 mM KOH, with two volumes of saliva or with two volumes of universal transport medium or Hanks' medium containing saliva. The resulting AG processing composition contained: 21.7% (v/v) PEG200, 67% (v/v) saliva, and 15 mM KOH, with pH ranging from 12.2 to 12.8 (depending pH of saliva specimens). AG processing was performed by incubating samples at room temperature for 5 to 30 min.

Direct RT-qPCR detection of SARS-CoV-2 RNA

An aliquot of 5 μl of the AG processed composition was added to 15 μl of RT-qPCR reaction mix; thus, each RT-qPCR reaction contained 3.33 μl of processed saliva. RT-qPCR was performed for 45 cycles (fast cycling) following the protocol specified for each manufacturer's kit. Reaction mixes were prepared using master mix, qPCR primer-probe mix, and Tris-HCl at pH 8 or 8.5, in accordance with the optimal conditions specified by each manufacturer. Where specified, the reaction mix included 0.2% Tween-20. Where indicated, some saliva samples were heated at 65 or 95 C prior to AG processing. Unless stated otherwise, each reaction was performed in triplicate and results are presented as the mean C_T value. Each reaction plate included control reactions: positive control reactions consisting of synthetic SARS-CoV-2 RNA or SARS-CoV-2 cDNA, processing composition reactions with no reference material, and reactions with neither processing composition nor reference material. Results were considered valid if all controls provided positive or negative results, respectively. A target was considered undetected for C_T values ≥ 40.

Results and discussion

The RNA genome of SARS-CoV-2, like other RNA viruses, is protected by a protein-lipid envelope [15, 16]. This envelope must be lysed to release RNA and make it available as template in molecular assays. A biological sample such as saliva contains carbohydrate and protein components, including ribonucleases, that degrade RNA and inhibit RT-PCR. The alkaline-glycol processing method developed in this study lyses virus particles and suppresses RT-PCR inhibitors. These combined actions allow for sensitive detection of viral RNA in saliva by direct RT-qPCR, without RNA purification. The high alkaline pH in AG processing is maintained by both a strong mineral base and glycols. In this report, AG processing was performed using a solution containing (v/v): 67% of saliva, 21.6% of polyethylene glycol 200 and 15-mM KOH. Depending on the initial pH of a saliva specimen, the pH during AG processing ranged from 12.2 to 12.8.

Table 1 compares C_T values for detecting SARS-CoV-2 RNA in human saliva by direct RT-qPCR performed with or without AG processing. Saliva samples were spiked with 10,000 copies of γ-irradiated or heat inactivated SARS-CoV-2 virus, or synthetic SARS-CoV-2 RNA. The N1 and N2 primer and probe sets amplified and identified target sequences in the nucleocapsid region of SARS-CoV-2 RNA. Both N1 and N2 primers amplified target sequences to a comparable degree, with the N1 primers performing marginally better. In compositions containing γ-irradiated or heat-inactivated virus, AG processing decreased the C_T for detecting SARS-CoV-2 RNA by about 5 amplification cycles, indicating a 32-fold increase in detection sensitivity. These results demonstrate detection of SARS-CoV-2 RNA in AG-processed human saliva and show that AG processing significantly increases the detection sensitivity for SARS-CoV-2 RNA.

Table 1. Detection of SARS-CoV-2 RNA in AG processed saliva by direct RT-qPCR.

Template material	Processing composition		N1 Target		N2 target	
			C_T	ΔC_T	C_T	ΔC_T
γ-irradiated SARS-CoV-2	Saliva	AG solution	24.16	5.91	25.09	5.82
		Water	30.07		30.91	
	Water	AG solution	23.28	5.96	24.24	5.64
		Water	29.24		29.88	
heat-inactivated SARS-CoV-2	Saliva	AG solution	25.07	4.68	26.90	4.90
		Water	29.75		31.80	
	Water	AG solution	27.34	9.45	29.46	8.99
		Water	36.79		38.45	
SARS-CoV-2 synthetic RNA	Saliva	AG solution	27.15	N/A	28.18	11.80
		Water	Undetected		39.98	
	Water	AG solution	26.33	-0.13	27.41	0.11
		Water	26.20		27.52	

AG processing of saliva and direct RT-qPCR were performed as described in Methods. AG processing compositions contained one volume of alkaline glycol solution (AG solution) plus two volumes of saliva or water. Saliva control processing compositions contained water, saliva and no alkaline glycol solution. Water control compositions contained water and no alkaline glycol solution nor saliva. Saliva was spiked with 1.00×10^4 copies of the indicated template material per RT-qPCR reaction. RT-qPCR was performed using Sigma KiCqStart One-Step Probe RT-qPCR ReadyMix ROX using reaction parameters following the manufacturer's protocol. C_T values report the mean of 3 replicates.

An even greater effect of AG processing was observed in tests using synthetic SARS-CoV-2 RNA as template. In AG-processed saliva, the synthetic viral RNA was detected at C_T 27, whereas without AG processing, viral RNA was undetectable using N1 primers and barely detectable using N2 primers. This result indicated that SARS-CoV-2 RNA in untreated saliva was rapidly degraded by saliva ribonucleases, rendering it unavailable as template for RT-qPCR. Collectively, the results in Table 1 suggest that AG processing increases the detection sensitivity for viral RNA by dual actions: it promotes release of RNA from the virus capsid, providing more usable RNA template for RT-qPCR, and it protects the RT-qPCR assay from inhibitory components in saliva.

It is notable that detection of viral RNA was not impaired by the high alkaline pH of AG processing (typically pH 12.2–12.8), which is known to fragment RNA. A likely explanation for this finding is that limited fragmentation of the large SARS-CoV-2 RNA genome during AG processing did not preclude effective detection of short RNA fragments. Most RT-qPCR products are less than 100 bp in length. Thus, limited fragmentation does not preclude detection of short amplicons such as the 71 bp N1 and 67 bp N2 sequences in the CDC SARS-CoV-2 test.

5 minutes of AG processing was sufficient for effective detection of SARS-CoV-2 RNA (Table 2). Extending AG processing time to 1 hour did not alter detection.

In addition to the Sigma KiCqStart kit used in Tables 1 & 2, AG processing can be used with other commercially available RT-qPCR kits (Table 3). The increase in sensitivity, measured as the decrease in C_T, ranged from 32- to 70-fold.

AG processing can be combined with a heating step prior to RT-qPCR and/or the addition of a non-ionic detergent to the RT-qPCR assay (Table 4). In our study, saliva containing inactivated virus was heated prior to AG processing and Tween-20 was added into the RT-qPCR reaction mix. Heating saliva for 30 min at 65 C or at 95 C substantially improved detection of viral RNA, as evidenced by a 5 C_T decrease in amplification cycles. Addition of Tween-20 to the RT-PCR reaction mix had a similar beneficial effect on detection of SARS-CoV-2 RNA. i.e.

Table 2. Effect of extended AG processing times on C_T values.

Processing time (min)	$C_T \pm SD$
5	26.69 ± 0.01
15	26.53 ± 0.03
30	25.79 ± 0.12
60	25.95 ± 0.02
120	26.62 ± 0.07

An AG processing composition, consisting of saliva, γ-irradiated SARS-CoV-2, and AG solution, was aliquoted and incubated at room temperature for 5 to 120 min prior to direct RT-qPCR. Each RT-qPCR reaction contained 2.92 x 10^4 copies of γ-irradiated SARS-CoV-2 template and used Sigma KiCqStart One-Step Probe RT-qPCR kit. C_T values report the mean of 3 replicates.

Table 3. Detection of SARS-CoV-2 RNA in AG processed saliva using various RT-PCR kits.

RT-qPCR Kit	Processing Composition	C_T	ΔC_T
Promega GoTaq	AG solution	30.82	4.89
	Water	35.71	
Thermo TaqPath	AG solution	24.91	7.20
	Water	32.11	
Quantabio qScript	AG solution	25.80	6.02
	Water	31.82	

Each RT-qPCR reaction contained 1.50 x 10^4 copies of heat-inactivated SARS-CoV-2 as template, and primer and probe sets for SARS-CoV-2 N1 target detection. C_T values report the mean of 3 replicates.

Table 4. Effect of Tween-20 and heat on C_T values for detecting SARS-CoV-2 in AG processed saliva.

Heat	Tween-20	C_T	Heat	C_T
30 min @ 95 C	0.2%		30 min @ 65 C	
-	-	30.09	-	31.81
-	+	26.31	-	24.44
+	-	24.92	+	26.26
+	+	24.94	+	23.95

Saliva specimens spiked with γ-irradiated SARS-CoV-2 were incubated for 30 min at 65 C or 95 C. After incubation, saliva specimens were AG processed and assayed by RT-qPCR using the KiCqStart One-Step RT-qPCR kit with the N1 primer and probe set. Each RT-qPCR reaction contained 1.00 x 10^4 virus copies. Some RT-qPCR assays were supplemented with Tween®-20 (CAS 9005-64-5) to reach 0.2% concentration in the reaction mix. Each C_T value reports the mean of 3 replicates.

either heating or addition of Tween-20, when applied separately, had similar beneficial effects on detection of SARS-CoV-2 RNA; however, their combined effects were not additive. When Tween-20 was present in the reaction mix, heating was not necessary to achieve the optimal SARS-CoV-2 detection sensitivity reported in this study. Nonetheless, heating clinical samples at >56 C to inactivate virus prior to handling can provide an additional layer of safety [17]. In additional experiments, Tween-20 added directly to saliva samples instead of the RT-qPCR reaction mix caused a time-dependent degradation of SARS-CoV-2 RNA (results not shown).
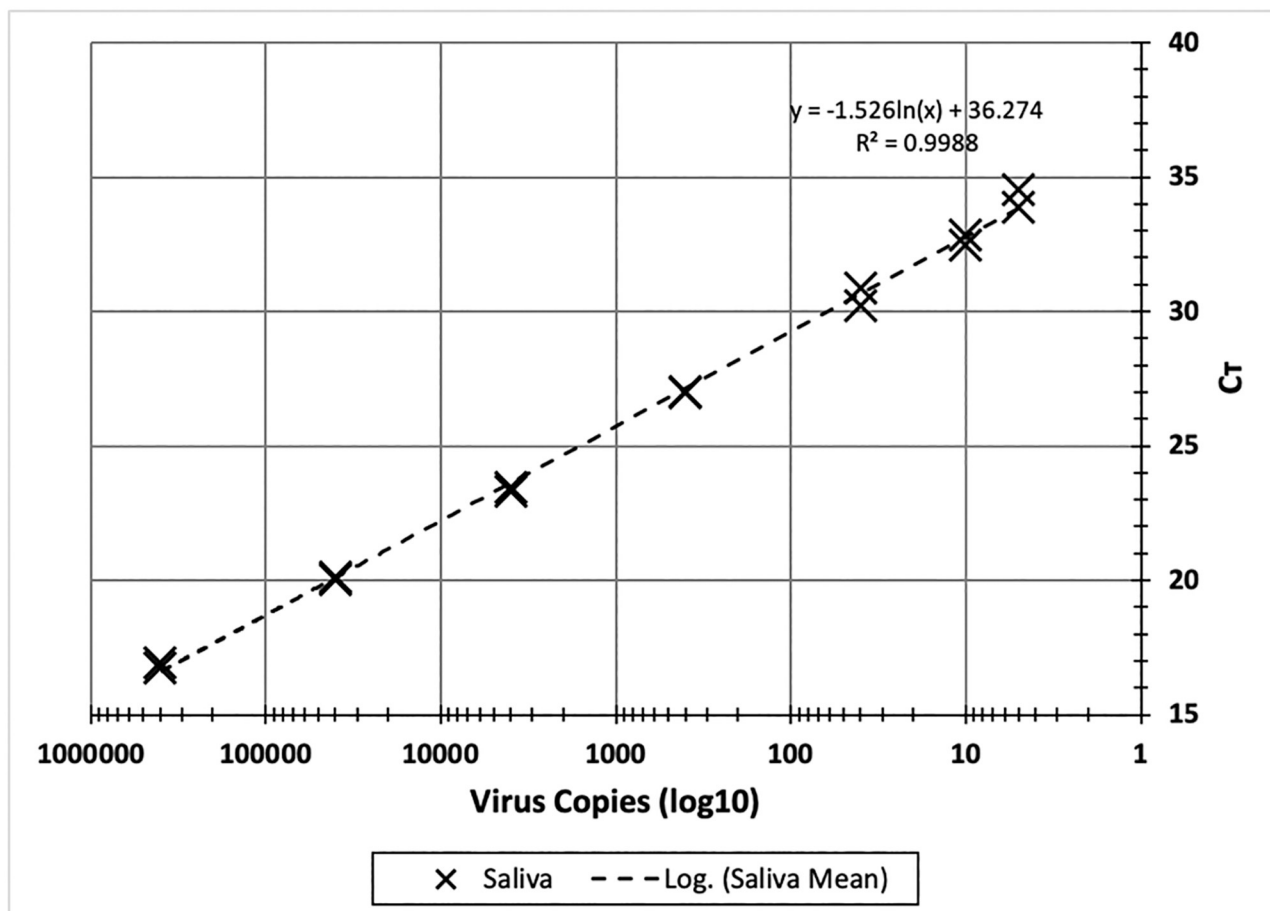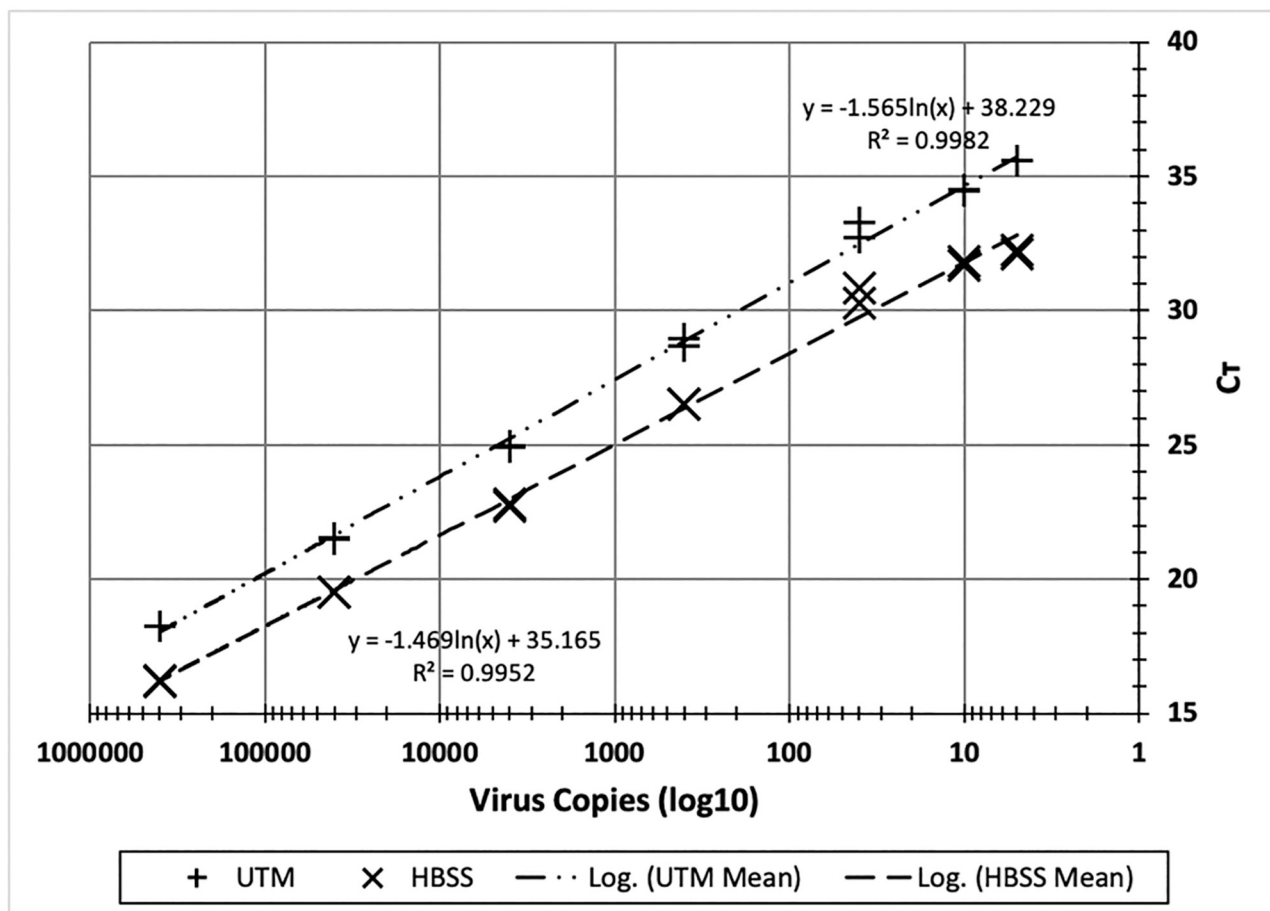
Fig 1. Detection range for SARS-CoV-2 in saliva using direct RT-qPCR with AG processing. AG processing solutions were prepared by mixing 2 volumes of saliva spiked with γ-irradiated SARS-CoV-2 and 1 volume of alkaline glycol solution. SARS-CoV-2 concentrations, per RT-qPCR reaction, ranged from 400,000 to 4 virus copies. RT-qPCR was performed with 1 step KiCqStart RT-PCR kit with primer and probe set for N1 target detection. C_T values show duplicate determinations at each concentration.

https://doi.org/10.1371/journal.pone.0246867.g001

AG processing of saliva with direct RT-qPCR allows a wide detection range (Fig 1). The detection range for γ-irradiated SARS-CoV-2 RNA in AG-processed saliva was from 400,000 copies to 4 copies per RT-qPCR reaction. C_T values ranged from 16.8 for 400,000 copies to 33.7 for 5 copies per reaction. Thus 400,000 and 4 copies per reaction correspond to 1.2×10^8 copies and 1.5×10^3 copies of viral RNA per ml of saliva, respectively.

Human saliva collected from infected subjects contains from 10^{10} to 10^4 SARS-CoV-2 virus particles per ml of saliva [5]. Thus, the method reported here–AG processing with direct RT-qPCR–provides an effective, sensitive, and robust diagnostic tool for detecting SARS-CoV-2 RNA.

It has been reported that saliva samples provide a more sensitive specimen for detecting SARS-CoV-2 in COVID-19 patients than nasopharyngeal swabs [5]. However, nasopharyngeal swabs are still a prevalent method of specimen collection for COVID-19 testing. Following collection, oropharyngeal or nasopharyngeal swab specimens are immersed in UTM or Hanks' medium and taken for COVID-19 testing. Specimen collection by swab does not provide a consistent amount of biological matter for diagnosis and significant variability in the number of cells and viral particles in nasopharyngeal specimens has been reported [18]. In our tests of

Fig 2. Detection range for SARS-CoV-2 in Universal Transport Medium for viruses (UTM) or Hanks storage medium (HBSS) using AG processing with direct RT-qPCR. Samples were prepared by mixing 2 volumes of UTM or Hank's containing 20% saliva and spiked with γ-irradiated SARS-CoV-2 with 1 volume of alkaline glycol solution. Final SARS-CoV-2 concentrations ranged from 4 to 400,000 viruses per RT-qPCR reaction. RT-qPCR was performed with 1 step KiCqStart RT-PCR kit with primer and probe set for the N1 target. C_T values report duplicate measurements.

buccal swabs, the amount of swab-collected material varied over 2-fold, from 60 mg to 170 mg of specimen per swab.

To simulate swab-derived clinical samples and provide a defined sample content for determining detection range, we prepared compositions containing γ-irradiated SARS-CoV-2 in UTM or Hanks' medium and 20% (v/v) saliva. The virus detection range for SARS-CoV-2 RNA was similar to that in AG-processed saliva (Fig 2). C_T values were 18.2 and 16.2 for 400,000 copies of SARS-CoV-2, and 35.5 and 32.2 for 4 copies of SARS-CoV-2 in UTM and Hanks' medium, respectively.

To determine the lower limit of detection (LOD) for SARS-CoV-2 RNA copy number in AG-processed saliva, we tested samples containing single-digit numbers of virus particles per reaction (Table 5). Saliva was spiked with SARS-CoV-2 virus to reach a final concentration of 3, 2, and 1 copy per reaction. Each viral concentration was tested in 20 repetitions. In accord with U.S. FDA guidelines, determination of LOD requires 19 positive detections out of 20 repetitions, and the FDA LOD is taken as twice the lowest detectable number of copies per mL of specimen. Results in Table 5 show 20, 19, and 19 positive RT-qPCR results for 3, 2, and 1 copy per reaction, respectively. The method detects 1 viral copy per reaction, which corresponds to an LOD of 300 viral copies per ml of saliva (600 copies/mL using the FDA definition).

Table 5. LOD for detecting SARS-CoV-2 in AG processed saliva by direct RT-qPCR.

SARS-CoV-2, copies per reaction	Successful repetitions (of 20)	$C_T \pm SD$	LOD, copies/mL
3	20	33.01 ± 0.90	
2	19	33.42 ± 0.89	
1	19	34.27 ± 1.04	**300**

AG processing solutions were prepared by mixing two volumes of saliva spiked with γ-irradiated SARS-CoV-2 and one volume of alkaline glycol solution, to contain 3, 2, or 1 viral particle(s) per RT-qPCR reaction, performed using the KiCqStart One-Step RT-qPCR kit with the N1 primer and probe set. Each reaction contained 3.33 μL of the initial saliva specimen.

https://doi.org/10.1371/journal.pone.0246867.t005

Table 6. LOD for detecting SARS-CoV-2 by direct RT-qPCR using AG processing of saliva in UTM or HBSS.

Sample Medium	SARS-CoV-2, copies per reaction	Enzyme Kit	Successful replicates (of 20)	$C_T \pm SD$	LOD, copies/mL
UTM	3	KiCqStart	20	36.98 ± 0.50	**900**
	2		17	37.64 ± 0.79	
	1		15	38.57 ± 1.05	
HBSS	3	KiCqStart	20	36.67 ± 0.84	
	2		20	37.25 ± 0.66	**600**
	1		18	37.64 ± 0.92	
UTM	3	TaqPath	20	36.41 ± 0.62	
	2		20	37.25 ± 0.59	**600**
	1		15	37.86 ± 0.98	
HBSS	3	TaqPath	20	37.72 ± 0.88	
	2		19	37.86 ± 0.85	**600**
	1		17	38.48 ± 0.63	

Specimens consisted of 20% saliva in UTM or HBSS, spiked with γ-irradiated SARS-CoV-2, and were heated at 65 C for 30 min prior to AG processing. AG processing solutions consisted of two volumes of specimen and one volume of alkaline glycol solution. Each RT-qPCR reaction contained 3, 2, or 1 viral copy and 3.33 μL of the initial specimen, and 0.2% Tween-20. RT-qPCR was performed using the N1 primer and probe set using either a KiCqStart One-Step RT-qPCR kit or TaqPath kit.

https://doi.org/10.1371/journal.pone.0246867.t006

The LOD for SARS-CoV-2 RNA copy number in simulated swab specimens (20% saliva in UTM or HSSB) is given in Table 6.

It should be noted that the detection sensitivity of AG processing with direct RT-qPCR can be increased an additional two-fold by increasing the RT-qPCR reaction volume from 20 μl to 50 μl (results not shown).

Finally, additional validation of our method was provided by the diagnosis of COVID-19 in three sample donors. The donors provided a self-collected saliva specimen that was incubated at 95 C for 30 min, then AG processed and used for detection of SARS-CoV-2 RNA by direct RT-qPCR with primers for the N1 target. Results showed amplification of the target sequence in the control samples without added reference material. In accordance with IRB requirements, these donors were notified of the results and instructed to consult a physician. They were diagnosed with COVID-19 following a positive result from an FDA-approved nasopharyngeal swab-based RT-PCR test.

Conclusions

AG processing combined with direct RT-qPCR is an effective and sensitive method for detecting SARS-CoV-2 without a requirement for viral RNA purification. The sensitivity of the method for detecting SARS RNA in AG-processed saliva is on par or greater than existing

Table 7. LOD of various molecular tests for detecting SARS-CoV-2 RNA in saliva.

Reference	saliva treatment	method	LOD, copies per mL saliva
[19]	saliva in UTM or VTM	RNA extraction, RT-qPCR	600–180,000
[7, 9, 10]	saliva into buffer, heat 95C/30 min, add Tween-20 to reaction/saliva?	direct RT-qPCR	1,000–14,000
This report	saliva into AG solution, 5 min	direct RT-qPCR	300 *

* LOD per FDA rule is 600 copies/mL

https://doi.org/10.1371/journal.pone.0246867.t007

methods that require an initial RNA extraction step (Table 7). Currently, all FDA reviewed kits that use saliva specimens require RNA purification, and their LODs range from 600 to 180,000 virus copies per mL of saliva [19]. Various methods for detecting SARS-CoV-2 in saliva without RNA extraction report LOD values from 1000 to 14,000 copies/mL [7, 9,10]. AG processing is effective over a wide range of viral concentrations in either saliva specimens or samples collected by swab into media. AG processing is robust across different RT-qPCR platforms and works with multiple FDA-approved RT-qPCR products. These advantages, plus its simplicity and cost-effectiveness, can increase laboratory throughput and streamline COVID-19 diagnosis. Although this report focused on the use of AG processing for detection of SARS-CoV-2 in saliva or swab-derived oral specimens, the method can also be used for processing SARS-CoV-2 containing specimens derived from a variety of biological fluids and tissues. It is also easily adapted for processing specimens containing other RNA viruses.

Author Contributions

Conceptualization: Piotr Chomczynski, Peter W. Chomczynski, Judith A. Heiny, Karol Mackey.

Data curation: Piotr Chomczynski, Peter W. Chomczynski, Amy Kennedy, Judith A. Heiny, Karol Mackey.

Formal analysis: Piotr Chomczynski, Peter W. Chomczynski, Amy Kennedy, Michal Rymaszewski, Judith A. Heiny, Karol Mackey.

Funding acquisition: Piotr Chomczynski.

Investigation: Piotr Chomczynski, Peter W. Chomczynski, Amy Kennedy, Michal Rymaszewski, William W. Wilfinger, Karol Mackey.

Methodology: Piotr Chomczynski, Peter W. Chomczynski, William W. Wilfinger, Karol Mackey.

Project administration: Piotr Chomczynski.

Supervision: Piotr Chomczynski, Karol Mackey.

Validation: Piotr Chomczynski, Peter W. Chomczynski, Amy Kennedy, Michal Rymaszewski.

Visualization: Peter W. Chomczynski.

Writing – original draft: Piotr Chomczynski, Judith A. Heiny.

Writing – review & editing: Peter W. Chomczynski, Judith A. Heiny, Karol Mackey.

References

1. CDC 2019-Novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel. Document #: CDC-006-00005 Revision #: 03. 3/15/2020

2. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. Clin Chem 2020; 66(4):549–55. https://doi.org/10.1093/clinchem/hvaa029 PMID: 32031583

3. Li Y, Li J, Zhang Y, Dai L, Li L, Liu J, et al. Development of an automatic integrated gene detection system for novel severe acute respiratory syndrome-related coronavirus (SARS-CoV2). Emer Microb Infect 2020; https://doi.org/10.1080/22221751.2020.1782774 PMID: 32543298

4. Park GS, Ku K, Baek SH, Kim SJ, Kim SI, Kim BY, et al. Development of reverse transcription loop-mediated isothermal amplification assays targeting severe acute respiratory syndrome coronavirus 2. J Mol Diagn 2020; https://doi.org/10.1016/j.jmoldx.2020.03.006 PMID: 32276051

5. Wyllie AL, Fournier J, Casanovas-Massana A, Campbell M, Tokuyama M, Vijayakumar P, et al. Saliva is more sensitive for SARS-Cov-2 detection in COVID-19 patients than nasaopharyngeal swabs. medRxiv 2020; https://doi.org/10.1101/2020.04

6. Bruce E.A, Huang ML, Perchetti GA, Tighe S, Laaguiby P, Hoffman JJ, et al. Direct RT-qPCR detection of SARS-CoV-2 RNA from patient nasopharyngeal swab without an RNA extraction step. PLoS Biol 2020; 18(10) e3000896. https://doi.org/10.1371/journal.pbio.3000896 PMID: 33006983

7. Ranoa DR, Holland RL, Alnaji FG, Green KJ, et al. Saliva-based molecular testing for SARS-CoV-2 that bypasses RNA extraction. bioRxiv 2020. https://doi.org/10.1101/2020.06.18.159434

8. Hasan MR, Mirza F, Al-Hail H, Sundararaju S, Xaba T, Iqbal M, et al. Detection of SARS-CoV-2 RNA by direct RT-qPCR on nasopharyngeal specimens without extraction of viral RNA. PLoS ONE 2020; 15(7): e0236564. https://doi.org/10.1371/journal.pone.0236564 PMID: 32706827

9. Wee SK, Sivalingam SP, Yap EPH. Rapid direct nucleic acid amplification test without RNA extraction for SARS-CoV-2 using a portable PCR Thermocycler. Genes 2020; 11, 664. https://doi.org/10.3390/genes11060664 PMID: 32570810

10. Vogels CBF, Watkins AE, Harden CA, Brackney D, Shafer J, Wang J, et al. SalivaDirect: A simplified and flexible platform to enhance SARS-CoV-2 testing capacity. mrdRxiv-BMJ Yale 2020; https://doi.org/10.1016/j.medj.2020.12.010 PMID: 33521748

11. Smyrlaki I, Ekman M, Vondracek N, Lentini A, DeSousa NR, Papanicolaou N, et al. Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-qPCR. Nature Comm 2020; 11 (1):4812. https://doi.org/10.1038/s41467-020-18611-5 PMID: 32968075

12. Chomczynski P, Rapid processing of biological samples for SARS-CoV-2 RNA detection. Patent pending, US63/117,776. US Patent Office, 2020.

13. Chomczynski P. Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. Biotechniques 2006; 40(4):454–458. https://doi.org/10.2144/000112149 PMID: 16629392

14. Chomczynski P. Reagents for storage and preparation of samples for DNA analysis. 2010, US Patent 7,727,718

15. Mandala VS, McKay MJ, Shcherbakov AA, Dregni AJ, Kolocouris A, Hong M. Structure and drug binding of the SARS-CoV-2 envelope protein in lipid bilayers. Res Sq 2020; https://doi.org/10.1038/s41594-020-00536-8 PMID: 33177698

16. Mousavizadeh L, Ghasemi S. Genotype and phenotype of COVID-19. J Microb Immun Infect 2020; https://doi.org/10.1016/j.jmii.2020.03.022 PMID: 32265180

17. Pastorino B, Touret F, Gilles M, Lamballerie X, Charrel RN. Heat Inactivation of Different Types of SARS-CoV-2 Samples: What Protocols for Biosafety, Molecular Detection and Serological Diagnostics? Viruses 2020; 12, 735; https://doi.org/10.3390/v12070735 PMID: 32646015

18. VanWesenbeck L, Meeuws H, D'Haese D, Ispas G, Houspie L, VanRanst M, et al. Sampling variability between two mid-turbinate swabs of the same patient has implications for influenza viral load monitoring. Virol J 2014; 11:233. https://doi.org/10.1186/s12985-014-0233-9 PMID: 25539740

19. FDA SARS-CoV-2 Reference Panel Comparative Data, 12-07-2020, Table 2C, www.fda.gov/medical-devices/coronavirus-covid-19-and-medical-devices/sars-cov-2-reference-panel-comparative-data#table2c)