ARTICLE

Saliva "Treat-and-Heat" Triplex Reverse Transcription Loop-Mediated Isothermal Amplification Assay for SARS-CoV-2

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The demand for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) molecular diagnostics that are faster, cheaper, and simpler to run than nasopharyngeal-based reverse transcription quantitative PCR (RT-qPCR) tests remains unmet in many parts of the world. In the Philippines, geographical and economic access to quality diagnostic testing remains out of reach for many communities. We describe the preclinical development of a fluorescence-based reverse transcription loop-mediated isothermal amplification test that uses drooled saliva as the biospecimen. Six treat-and-heat ("direct") procedures that inactivate the virus and release the target RNA were compared. Using duplexed As1e and E1 primers, protocols derived from Ben-Assa *et al.* (2020) using proteinase K or from Rabe and Cepko (2020) using TCEP (Tris(2-carboxyethyl)phosphine hydrochloride)/EDTA provided reliable RNA amplification. The TCEP/EDTA-based method in particular showed improvement in robustness in duplex *vs.* singleplex format. Inclusion of human β-actin primers provided a triplex test with an internal amplification control that could be distinguished from SARS-CoV-2 amplicons based on melt curve analysis. After including the dUTP/uracil-DNA glycosylase system and implementing laboratory procedures to avoid cross-contamination, false positive amplification was acceptably rare. The duplex or triplex tests are predicted to reliably detect patient salivary viral loads >100 copies/μL and to yield equivocal results between 10 and 100 copies/μL. These viral loads, corresponding to RT-qPCR C_t ~29–32, are expected to identify the majority of infected and, particularly, of infectious patients. If clinically validated, the test would provide additional testing capacity requiring only a fraction of the time, cost, and infrastructure of the current nasopharyngeal swab–based RT-qPCR test, thereby improving access to testing for more Filipinos.

Key Words: fluorescence, melt curve, extraction-free, Proteinase K

INTRODUCTION

The global pandemic caused by the coronavirus disease 2019 has led to an unprecedented demand for diagnostics for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). More than a year on, viral detection from nasopharyngeal swabs (NPSs) using reverse transcription quantitative PCR (RT-qPCR) remains the reference diagnostic method. Although highly sensitive, the complex NPS–RT-qPCR workflow limits accessible testing at scale, particularly in the more remote parts of a low-resource archipelago like the Philippines. Specifically, addressing the requirements for nucleic acid extraction, expensive real-time fluorescence thermocyclers, and highly trained laboratory personnel remains a challenge in the country. Thus, in order to meet local demand for coronavirus disease 2019 diagnostics, there is a pressing need to simplify the RT-qPCR workflow. Reverse transcription loop-mediated isothermal amplification (RT-LAMP)[1,2] is a simpler and thus attractive alternative method with detection sensitivities that can approach those of RT-qPCR.[3–5]

To avoid the invasive and uncomfortable collection of NPSs, anterior nares swab, gargle wash, and saliva have been investigated as potential biospecimens.[6–11] Following approval of saliva-based RT-qPCR testing, counterpart saliva-based RT-LAMP tests have been developed (reviewed in Tan *et al.*[6]). Finally, RNA extraction represents a financial, supply-chain and throughput bottleneck, leading to development of various "extraction-free" processing techniques. Such methods are divided broadly into those using proteinase K (*e.g.*, Vogels *et al.*,[8] Ben-Assa *et al.*,[12] Lalli *et al.*[13]) and those using >75°C heating and nonenzymatic chemical extraction (*e.g.*, Rabe and Cepko[14]; Yu AD, Galatsis K, Zheng J, *et al.*, unpublished results; Ranoa DRE, Holland RL, Alnaji FG, *et al.*, unpublished results). Proteinase K, sometimes in combination with mild detergents, digests the encapsulating viral proteins and inhibitory proteins/RNases that can reduce amplification efficiency; it can

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https://doi.org/10.7171/jbt.21-3203-014

also help to reduce saliva viscosity and therefore aid pipetting.[15] In the nonenzymatic protocols, combinations of a reducing agent (Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)) and a divalent metal ion chelator (EDTA) reduce the viscosity of the saliva and inhibit RNases.

Here we compare several reported extraction-free saliva-based methods for RT-LAMP, with and without proteinase K, measuring assay specificity and sensitivity on contrived samples with As1e and E1 primer sets. Duplex As1e-E1 tests with the best performing extraction method in each category[12,14] were augmented with β-actin (ACTB) as an internal amplification control. Both duplex and triplex methods had good specificity, an acceptable sensitivity (10–100 copies/μL saliva), and the optional detection of an internal control amplicon in triplex format.

MATERIALS AND METHODS

Loop-Mediated Isothermal Amplification and PCR Primers

Unmodified macrogen oligonucleotide purification cartridge–purified primers were from Macrogen (South Korea). ORF1ab (As1e), envelope (E1), and human ACTB primer sequences were from Zhang *et al.*[3] and Kellner MJ, Ross JJ, Schnabl J, *et al.* (unpublished results; https://doi.org/10.1101/2020.06.23.166397). In a singleplex format, the concentration of each SARS-CoV-2 primer was 1.6 μM forward/backward inner primer, 0.2 μM outer forward (F3)/outer backward (B3), and 0.4 μM loop forward/backward; in multiplex formats, each primer concentration was halved. Primers for the ACTB-positive amplification control (Zhang *et al.*[3]; Anahtar *et al.*[16]; Kellner *et al.*, unpublished results) were used at 20% of the concentration of other primers: 0.32 μM forward/backward inner primer, 0.04 μM F3/B3, and 0.08 μM loop forward/backward. For RT-qPCR, N gene primer and probe sequences were from the Centers for Disease Control and Prevention[17] and used at 0.5 μM and 0.2 μM, respectively.

Control RNA

The SARS-CoV-2 RNA template was from BEI Resources (Manassas, VA, USA): quantitative PCR (qPCR) control RNA from heat-inactivated SARS-CoV-2 isolate (USA-WA1/2020; BEI-52347, Lot #70033926) and SARS-CoV-2 γ-irradiated virus (GIV) (isolate USA-WA1/2020; BEI-52287; Lot #70035888). Their provenance with respect to storage temperature during importation through Customs remains uncertain (see below). The RNA controls needed to be stored at −20°C in small volume aliquots. Serial dilutions were diluted in either water or TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.0) as indicated with 1 U/μL RNAse Inhibitor. Human total RNA was extracted from human embryonic kidney (HEK) cells with TRIzol and stored at −20°C.

Collection and Processing of Saliva Samples

Clear saliva (drool) samples were obtained with informed consent from healthy control subjects according to guidelines outlined by Tan *et al.*[6] and stored at 4°C for no more than 1 h before either being processed or stored in liquid nitrogen. *Before processing*, saliva samples were treated with its respective buffer (Table 1) and spiked (0.5–1% v/v) with 2-fold serial dilutions of GIV to yield a notional final spiked concentration of 1.6–200 copies/μL saliva. These were used as surrogate patient samples and subjected to various processing methods as described below. Each 20 μL RT-LAMP test included 5 μL of input processed saliva containing the indicated number of spiked viral copies. For the

TABLE 1

Comparison of inactivation and extraction methods

Method	Principle	Addition(s)	Heating	$[saliva]_{final}$ (% v/v)	Reference
[A]	Enzymatic	50 μL saliva + 6.25 μL 20 mg/mL ProK (final: 2.2 mg/mL)	1 min @ RT 5 min @ 95°C	22	Vogels *et al.*[8]
[B]		5 μL saliva + 40 μL 1.4 mg/mL ProK (final: 1.24 mg/mL)	15 min @ RT 5 min @ 95°C	2.8	Ben-Assa *et al.*[12]
[C]		50 μL saliva + 50 μL 2× PBS-0.4 mg/mL ProK (final: 0.2 mg/mL)	15 min @ 65°C 5 min @ 95°C	12.5	Lalli *et al.*[13]
[D]	Non- enzymatic	50 μL saliva + 50 μL 5 mM TCEP/2 mM EDTA, 2 U/μL RNase I	10 min @ 95°C	12.5	Rabe and Cepko[14]
[E]		50 μL saliva + 50 μL 12.5 mM TCEP, 2 mM EDTA, 2 U/μL RNase I	5 min @ 95°C	12.5	Yu *et al.*, unpublished results
[F]		50 μL saliva + 50 μL 2× TBE/2% v/v Tween 20 (after heating saliva)	30 min @ 95°C	12.5	Ranoa *et al.*, unpublished results

ProK, proteinase K; RT, room temperature; TBE, Tris-borate-EDTA.

nontemplate control (NTC), nuclease-free water was added as a sample. For convenience, heating and cooling steps were performed in 0.2 mL PCR tubes using a thermocycler (G-Storm Thermocycler, GS00482, Sigma-Aldrich, USA). Saliva was either processed in bulk, then aliquoted and stored at −20°C, or prepared freshly and used immediately; no discernable difference was observed between results from using the 2 procedures. Six extraction-free saliva processing methods were taken, or adapted, from the literature (Table 1).

Enzymatic Protocols

Three protocols are based on proteinase K. **[A]**: In the SalivaDirect procedure as applied to RT-qPCR (Vogels *et al.*[8]), GIV-spiked saliva was briefly vortexed until homogenous. Fifty microliters were transferred to a tube containing 6.25 μL of proteinase K (20 mg/mL, final concentration of 2.2 mg/mL, P8170S, New England Biolabs Singapore). The samples were vortexed for 1 min and incubated at 95°C for 5 min before storing at 4°C. **[B]**: The protocol by Ben-Assa *et al.*[12] as applied to NPS/Viral Transport Media (VTM) was adapted for use with saliva. Five microliters of saliva was added to 2.8 μL of 20 mg/mL proteinase K (New England Biolabs, Ipswich, MA, USA) in 37.2 μL of nuclease-free water, incubated at 25°C for 15 min, and heated at 95°C for 5 min before storing at 4°C. **[C]**: Equal (50 μL) volumes of saliva and 2× phosphate-buffered saline, pH 7.5, were added to 1 μL of proteinase K (final concentration 0.2 mg/mL), incubated at 65°C for 15 min, heated at 95°C for 5 min, and stored at 4°C (Lalli *et al.*[13]).

Nonenzymatic Protocols

[D]: The method by Rabe and Cepko[14] was modified with the addition of 1 U/μL RNase inhibitor (M0314L, New England Biolabs Singapore). Equal volumes (typically 500 μL) of saliva and an inactivation solution (5 mM TCEP, 2 mM EDTA, 29 mM NaOH, 2 U/μL RNase inhibitor) were incubated at 95°C for 10 min before storing at 4°C until use. This method (addition of 50% v/v 2× TCEP/EDTA) differs from the original publication (addition of 1% v/v 100× TCEP/EDTA) to reduce viscosity, aid pipetting, and sample manipulation. **[E]**: A similar method (Yu AD, Galatsis K, Zheng J, *et al.*, unpublished results; https://doi.org/10.1101/2020.12.26.20248880) was also modified to include RNase inhibitor (1:1 v/v saliva: 12.5 mM TCEP, 2 mM EDTA, 29 mM NaOH, 2 U/μL RNase inhibitor) with 95°C heating for 5 min. **[F]**: Developed for RT-qPCR, the method of Ranoa DRE, Holland RL, Alnaji FG, *et al.* (unpublished results; https://doi.org/10.1101/2020.06.18.159434) involves heating saliva at 95°C for 30 min *followed by* addition of an equal volume of 2× Tris-Borate-EDTA containing 2% w/v Tween 20.

Fluorescent RT-LAMP

Loop-mediated isothermal amplification (LAMP) reactions were performed using New England Biolabs WarmStart LAMP Kit (E1700L) as per the manufacturer's protocol. Prepared on ice, 10 μL mastermix (containing WarmStart RTx and Bst 2.0 DNA polymerases) was mixed with the indicated concentration of 6 LAMP primers per target, 40 mM guanidine hydrochloride (pH 8), 1 μM SYTO9 DNA intercalating dye (from a working stock in 11% v/v DMSO, S34854), 700 μM dUTP (in addition to the 1.4 mM of each deoxyribonucleotide triphosphate in the mastermix), and 0.2 U/μL Antarctic thermolabile uracil-DNA glycosylase (UDG; M0372L, New England Biolabs Singapore). Five microliters of processed saliva samples were added to 15 μL reaction mix (20 μL total volume) with 10 μL of mineral oil added into the reaction (to further minimize cross-contamination). Reactions were performed in either 0.2 mL PCR tubes (P-02-C, Extragene, Taiwan) or PCR plates (PC10HS-9-LP-N-AB; Gene Era Biotech, Hangzhou, China). In a CFX96 qPCR reader (Bio-Rad, Hercules, CA, USA), the RT-LAMP reactions were incubated at 25°C for 5 min to enable UDG digestion of contaminating DNA (Kellner *et al.*, unpublished results; Hsieh *et al.*[18]) followed by isothermal incubation at 65°C for 30 min (120 read cycles at 15 s intervals) and 5 min at 90°C to terminate the reaction by enzyme denaturation. A melt curve was generated in 0.2°C intervals between 75 and 98°C, encompassing melting transitions for the 3 amplicons (As1e: 84°C, E1: 86°C, ACTB: 92°C).

RT-qPCR

Reactions were performed using a Bio-Rad CFX96 qPCR with the New England Biolabs Luna Probe One-Step RT-qPCR kit (E3007E) following the manufacturer's instructions. Prepared on ice, reactions contained the New England Biolabs Luna Probe One-Step Reaction, SYTO 9 (1 μM, 0.44% v/v DMSO), Luna WarmStart RT enzyme mix, 500 nM primers (either N1 per the Centers for Disease Control and Prevention[17] or E1 F3/B3; see below), nuclease-free water, and 5 μL of sample to 20 μL total reaction volume. Reactions were layered with 10 μL of mineral oil before amplification (reverse transcription: 55°C for 10 min; denaturation: 95°C for 1 min, denaturation: 95°C for 10 s, annealing-extension one-step: 60°C for 30 s; 50 cycles). Amplicons were detected by an increase in SYTO 9 fluorescence (fluorescein amidite (FAM) channel), by characteristic −d(relative fluorescence units)/d(temperature) melt peaks (0.3°C intervals), and by 3% w/v agarose gel electrophoresis.

RESULTS

Use-case and target profile Our objective was to develop a saliva-based extraction-free RT-LAMP method, for use as

an alternative to RT-qPCR testing, in an established clinical pathology laboratory (*e.g.*, a secondary or tertiary hospital). This initial use-case differs from the low-infrastructure, visual readout, distributed diagnostics use-case often associated with LAMP, particularly in low- and middle-income countries. The target sensitivity is 100 copies/μL patient input saliva, representing a viral load that reflects the transition to higher infectivity.[19] *Primers and matrices* Primer sets were chosen based on their literature precedent and on pilot screening leading to the selection of As1e, E1, and ACTB as target genes (Zhang *et al.*,[3] Rabe and Cepko,[14] Anahtar et al.,[16] Dudley *et al.*[20]; Kellner *et al.*, unpublished results). Color N and N2 primer sets unexpectedly led to significant nontemplate amplification, whereas As1e and E1 were viable alternatives with acceptable sensitivity but without nonspecific amplification. ACTB proved a reliable human amplification control. All reactions were performed at 65°C with comparatively minor changes observed between 60 and 65°C (data not shown).

During assay development, we initially used intact GIV added to saliva *after* heat processing, with the most promising methods progressing to spiking *before* heat processing. The latter represents the closest surrogate of a patient sample. Methods were progressed or rejected based on sensitivity (reliable detection of target RNA within 15 min) and specificity (frequency and timing of mispriming, nontemplate amplification). Method development progressed initially from singleplex and then to a combined gene format (*i.e.*, multiple genes amplified per reaction though with only a single wavelength readout).[3,21–23]

Singleplex

Primer set sensitivity and specificity with purified RNA

When RNA purified by BEI Resources (BEI-52347) was serially diluted in RNase-free water, we observed robust detection of target RNA down to a *notional* RNA concentration of 63 copies per reaction (Fig. 1; 12.5 copies/μL input) with amplification becoming stochastic and unreliable at lower concentrations. We emphasize that these values likely overestimate the true or active concentrations—out of necessity the RNA and GIV were stored at -20°C rather than -80°C for >6 mo, during which time we observed a progressive decline in sensitivity (and increase in RT-qPCR C_t value; see below). Therefore, we quote concentrations with this caveat—the true concentrations might be somewhat lower and the sensitivities somewhat higher. That notwithstanding, repeated experiments yielded a median sensitivity of 63 copies per reaction for both primer sets (N = 5–7; Table 2). Robust amplification of RNA extracted from an HEK cell line was observed with an ACTB primer set (Fig. 1C). Data from this simplest of systems served as a benchmark for the succeeding saliva-based LAMP experiments.

Unsurprisingly for a laboratory that was new to LAMP, we initially encountered challenges with cross-contamination. These were addressed with increasingly stringent laboratory procedures, frequent autoclaving, avoiding running gels or opening postamplification reactions, physically separating addition of spiked virus into reactions from the addition of water as the NTC, ensuring the completeness of plate and tube sealing, and where possible using single use-and-discard aliquots of reagents and controls. These operating procedures were supplemented with the addition of the dUTP/UDG system (Hsieh *et al.*[18]; Kellner *et al.*, unpublished results) and addition of mineral oil, the latter arguably being unnecessary. With these changes, amplification in negative control reactions dramatically decreased; where seen, melt analysis indicated they arose from mispriming intrinsic to the reaction rather than extrinsic cross-contamination.

Method performance in spiked saliva

All 6 methods involve addition of either chemical reagents (TCEP/EDTA/detergents) or proteases, followed by heating. To create contrived patient samples, GIV can either be spiked into saliva *before* or *after* the reagent addition and heating step. In the latter case, RNases are largely inactivated prior to spiking, which can potentially overestimate test sensitivity because RNA release and RNase inactivation occur contemporaneously when using *bona fide* samples (Qian *et al.*, 2020). Therefore, the majority of our work and all the results below involve spiking of virus *before* processing, thereby more closely resembling the clinical samples. Methods were compared based on (1) time to positive (min), the counterpart of C_t in PCR, and (2) the frequency of false positives in the NTC reactions. These usually occur after 15 min (60 cycles) due to mispriming, often giving rise to shallow or pseudolinear amplification curves that almost always result in atypical melt profiles. As others have observed, the time to positive *vs.* input RNA relationship is compressed, reflecting amplification that is more binary than gradual as seen in qPCR. *Enzymatic methods* Each processing method exhibits high specificity indicating no or minimal off-target amplification of RNA/DNA released from human saliva (in Fig. 2A, a false positive is indicated by a hollow square). In singleplex format, As1e performed more reliably across methods than E1. Of the proteinase K methods, **[B]** (Ben-Assa *et al.*) was able to reliably detect 63 copies per reaction in saliva, notably below that using the Vogels *et al.* **[A]** procedure (Fig. 2A); both methods were qualitatively superior to **[C]** (Lalli *et al.*). Although both **[A]** and **[B]** are straightforward, the Ben-Assa *et al.* protocol

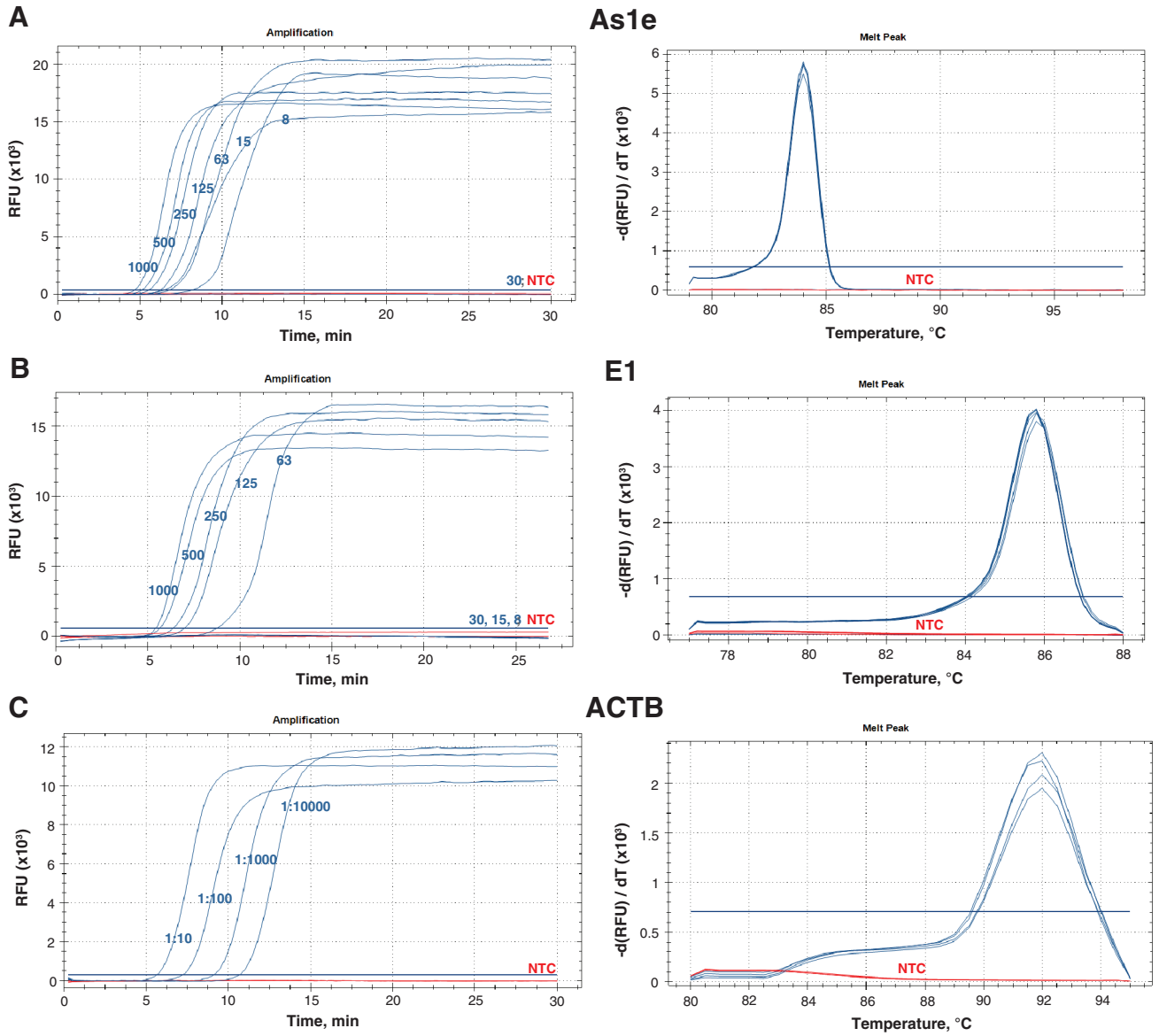

FIGURE 1

Amplification and melt profiles of purified SARS-CoV-2 and human RNA using As1e, E1, and ACTB primers. *A*, *B*) Values indicate notional RNA copies per reaction (see text). *C*) Serially diluted HEK cell line RNA. Both primer sets yielded reliable detection down to 63 copies per reaction (~12.5 copies/μL). The melt peaks are 84°C, 86°C, and 92°C for As1e, E1, and ACTB, respectively. Note that the E1 amplicon melt peak is largely symmetrical (*cf.* Fig. 3) and that the shoulder/plateau between 82 and 88°C is an intrinsic property of the ACTB amplicon (*cf.* Fig. 5). RFU, relative fluorescence units; T, temperature.

[B] uses a lower v/v final concentration of saliva in the LAMP reaction (~3% *vs.* ~20%). Saliva is a complex and viscous matrix such that a simple aqueous dilution has been reported to improve detection.[13] Necessarily, the larger dilution in **[B]** compared with **[A]** will partly offset any improvements in sensitivity in the reaction. However, using these and other primer sets (data not shown), we found more experiment-to-experiment and preparation-to-preparation variability with **[A]**. We note that Vogels *et al.* used a high concentration (and hence viscosity) of saliva without a reducing agent (*e.g.*, TCEP; see below). Therefore, we elected to progress method **[B]** by Ben-Assa *et al.* based on acceptable sensitivity and the most consistent between-run results among the enzymatic methods. *Nonenzymatic methods* All methods again yielded good specificity with rare (and, where observed, clearly distinguishable) late mispriming amplification in the negative control reactions. The modified Rabe and Cepko protocol **[D]** was the

TABLE 2

Summary of lowest detectable concentration of viral RNA. A series of dose-response curves (8–1000 copies per reaction) as outlined in Fig. 4 were used to determine the concentration of RNA below which amplification occurred after 15 min. Dose-response curves were generated on N test occasions from which the mean and median values were derived. We do not suggest statistical significance between data sets, which are only intended to be qualitative/semiquantitative.

Matrix	Buffer		Saliva				
Method	Purified RNA		Method [B]		Method [D]		
Reaction format	Mean ± SD	Median (N)	Mean ± SD	Median (N)	Mean ± SD	Median (N)	Figure
Singleplex (E1)	99 ± 93	63 (5)	40 ± 21	30 (4)	109 ± 104	140 (4)	1
Singleplex (As1e)	77 ± 87	63 (7)	47 ± 23	63 (5)	172 ± 94	250 (5)	1
Duplex (As1e+E1)	N.D.	N.D.	52 ± 42	46 (6)	35 ± 23	31 (6)	2, 3
Triplex (As1e+E1+ACTB)	N.D.	N.D.	33 ± 19	30 (9)	51 ± 44	30 (9)	4

N.D., not determined.

most sensitive, reliably detecting 63 (As1e) and 250 (E1) copies per reaction (Fig. 2). TCEP and EDTA effectively inactivate and inhibit RNases, whereas the reducing agent also limits sample viscosity, aiding accurate and reliable pipetting.[14] Although others have observed an improvement in sensitivity when including RNase inhibitors during sample preparation,[24] we observed relatively marginal improvements in sensitivity (2-fold), although it did noticeably aid reproducibility within and between experiments (data not shown). Addition of RNase inhibitors is therefore not essential but may be beneficial. Similarly, we did not observe the pronounced improvement in sensitivity reported by Yu *et al.* (unpublished results) for protocol **[E]** when increasing the TCEP concentration from 2.5 mM to 6.5 mM. In our experience, increasing the 95°C heating time to 10 min (*vs.* the 5 min of Yu *et al.*) provided the most consistent and repeatable extraction; this seems to be more important than the absolute TCEP concentration. Protocol **[F]** by Ranoa *et al.* (unpublished results), although validated for RT-qPCR, performed poorly with RT-LAMP (Fig. 2B), possibly due to the additional Tween 20 carried over into the RT-LAMP reaction.

In conclusion, using primer sets in singleplex format, we progressed protocols **[B]** and **[D]** as representatives of enzymatic and nonenzymatic saliva processing, respectively. We have observed similar results to those described here using different primer sets, which adds to the aggregate confidence in our conclusions despite the relatively small data sets presented herein.

Duplex

Amplifying multiple primer sets in one reaction can improve detection of SARS-CoV-2 by increasing genomic coverage (making the test less reliant on one primer set) and/or by reducing the limit of detection.[25] Duplexing with As1e/E1 showed no evidence of amplification in the

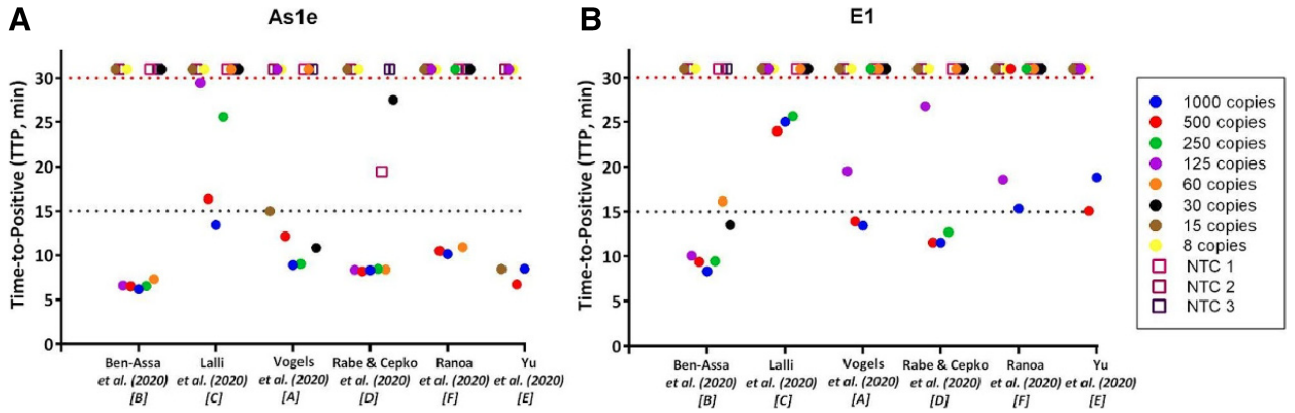

FIGURE 2

Time-to-positive (TTP) values with As1e and E1 primers in singleplex format using various extraction-free saliva processing protocols. Saliva samples were spiked with GIV prior to processing. Three NTC replicates were included in each protocol. The black and red broken lines correspond to the threshold and total reaction time, respectively. Reactions not producing amplification in 30 min are shown above the red broken line. Representative data of repeats (N = 4–5) is shown.

NTC samples that may have arisen from cross-primer mispriming (Fig. 3). However, we equally did not observe any marked improvement in sensitivity when using protocol **[B]** (duplex median = 46 copies per reaction; Table 2; Fig. 4B), although method **[D]** appeared to perform more robustly with 2 primer sets combined (duplex sensitivity ~31 copies per reaction; Table 2; Fig. 4C). That minimal improvements in sensitivity were observed might not be unexpected. In order to minimize potential for cross–primer-dimer formation (and hence false positives) the primers were used at 50% of the typical singleplex concentrations (so that the total primer concentration was unchanged). In the duplex, the E1 gene was preferentially amplified (by melt area under the curve) in ~85% of reactions; the remainder had amplification of both genes or, rarely, of As1e alone.

In summary, after implementing procedures to minimize cross-contamination, we were able to reliably achieve specific detection of As1e and E1 genes in saliva without RNA extraction down to ~50 GIV copies per reaction (~10 copies/μL) in the duplex format with both enzymatic and nonenzymatic methods.

Triplex

Including a human internal control primer set for ACTB led to a partial loss in sensitivity, as more of the limiting reagents (enzyme, deoxyribonucleotide triphosphate) were consumed in amplifying the control rather than the viral amplicon(s). ACTB primers at their standard concentrations reduced sensitivity for viral genes to >500 copies per reaction (data not shown). We therefore desensitized the ACTB amplification pathway by reducing the concentration of 6 primers *en masse* or by excluding loop primers.[26] We were unable to reliably delay the amplification of ACTB into a characteristic later time window (*e.g.*, 20–30 min *vs.* 5–15 min for SARS-CoV-2 amplification; data not shown). Nevertheless, robust and reliable amplification of all 3 As1e, E1, and ACTB genes was observed between 10 and 20 min at 30%, 25%, 20%, and 15% of the default ACTB primer concentration (data not shown), and we selected 20% ACTB primer for further experiments.

In triplex format ([As1e, E1] = 50%; [ACTB] = 20%), robust amplification is observed within 15 min in all reactions indicating amplification minimally of the ACTB gene with methods **[B]** and **[D]** (Fig. 5). Despite the differing (~3% v/v *vs.* ~13% v/v) test concentrations of saliva, both methods yielded comparable and robust ACTB amplification. At viral concentrations above 250 copies per reaction, 3 genes are amplified, E1 again being preferentially amplified over As1e (by melt area under the curve; see also Fig. 3). In the absence of SARS-CoV-2, only the ACTB amplicon is observed, as expected of an internal positive control. In this triplex format, a true *positive* is one with an ACTB melt peak between 90 and 95°C and SARS-CoV-2–derived melt peaks between 81 and 87°C (Fig. 5; blue curves); a SARS-CoV-2 true *negative* has only the ACTB peak at 92°C (Fig. 5; green curves); a false *negative* test would contain SARS-CoV-2 RNA but lack the 83–86°C peak (Fig. 5; red curves), whereas an *invalid* test (to be discarded or repeated) would have no detectable amplification of ACTB.

In summary (Table 2; Fig. 4), protocol **[B]** yielded relatively robust detection of viral RNA (median: 30 copies per reaction, range: 8–63, N = 9; *cf.* ~63 copies per reaction in a duplex). Although the median sensitivity for method **[D]** was also 30 copies per reaction, the interexperimental range appeared wider (15–125 copies per reaction, N = 9), which may reflect the impact of high viscosity and/or the higher saliva (and hence "competing" actin RNA) concentration during amplification.

Correcting for the test concentration of saliva (~3% v/v **[B]** or ~13% v/v **[D]**) and the input sample volume (5 μL), a *median* of 30 copies in the reaction corresponds to a viral load in the patient saliva of ~10 copies/μL and ~50 copies/μL for the methods by Rabe and Cepko and Ben-Assa *et al.*, respectively. More conservatively, if the *upper limit* of the range in Fig. 4 is used to better reflect variability, the corresponding patient saliva levels increase to ~50 copies/μL (**[D]**) and ~100 copies/μL (**[B]**). In summary, these preclinical data predict that both duplex and triplex formats would be able to detect the target viral load (≥100 copies/μL) in patient saliva using either an enzymatic (**[B]**) or nonenzymatic (**[D]**) method.

Secondary Reverse Transcription PCR Assays

The F3 and B3 LAMP primer pairs can serve as forward and reverse primers in (reverse transcription)–PCR, allowing an orthogonal (complementary) test for the presence of target gene or during assay design.[27,28] All 3 As1e, E1, and ACTB F3/B3 primer pairs generated amplicons of the expected size [E1: 210 base pairs (bp); As1e: 197 bp; ACTB: 212 bp] with viral and human RNA at relatively low concentrations but with poor efficiency (Fig. 6A). For E1 and ACTB F3/B3 primers, nontemplate primer-dimer amplification was comparatively rare, separated from target amplification temporally and with characteristic melt curves (Fig. 6A; main and inset). In contrast, As1e F3/B3 primers yielded problematic nonspecific amplification (Fig. 6A; inset and data not shown). At least for 3 primer sets, reverse transcription PCR amplification with F3/B3 is viable for qualitative confirmatory purposes (if no other primers are available) but is clearly far from ideal.

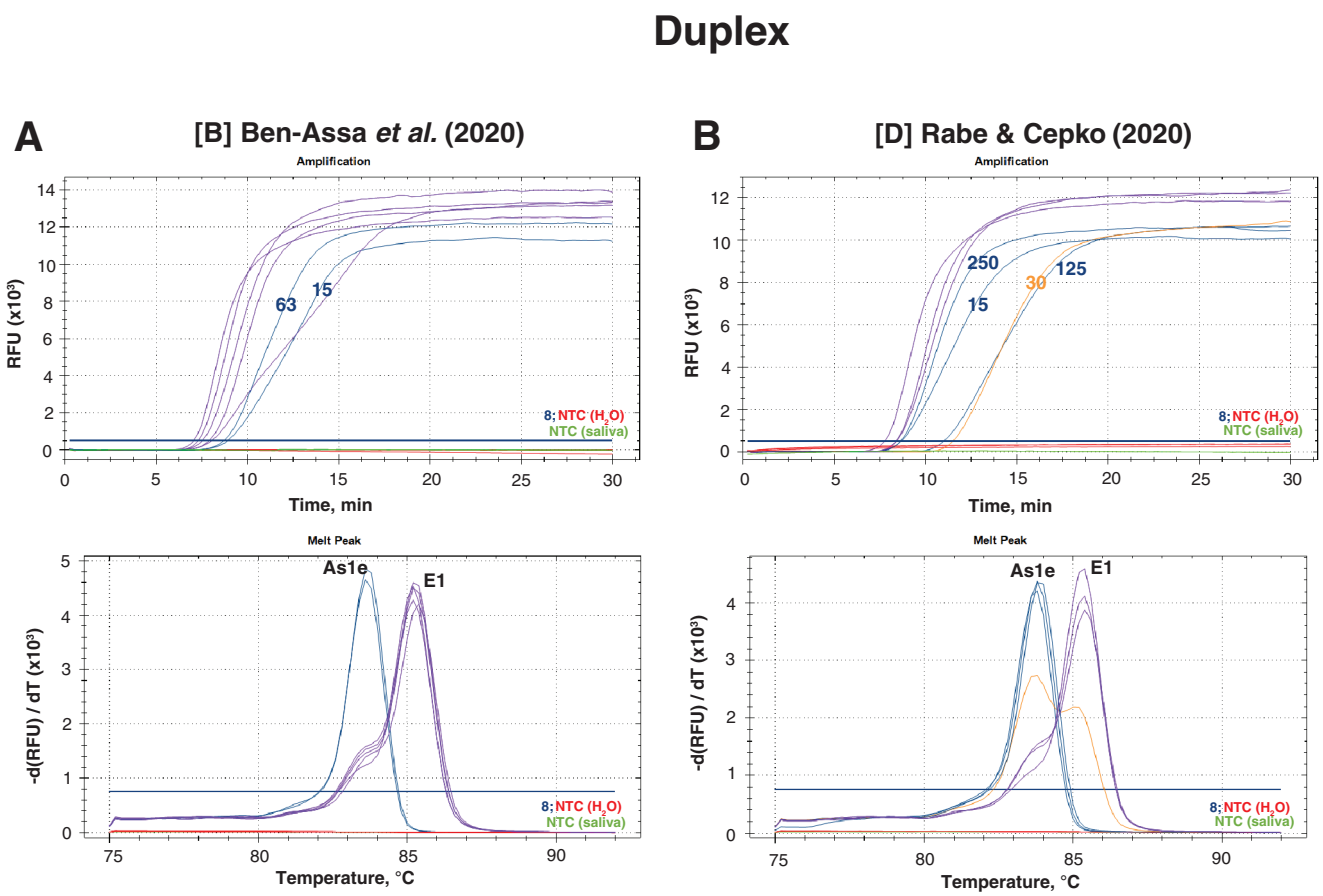

FIGURE 3

Amplification with duplexed primer sets (As1e and E1) using virus spiked into saliva. Blue: amplicons that contain only one gene (either As1e or E1); violet: amplicons with E1 > As1e estimated by melt area under the curve; orange: amplicons with E1 ≈ As1e; green: saliva negative controls without SARS-CoV-2 GIV; red: water negative controls. Samples with 8 copies per reaction did not amplify within 30 min. Note the low-temperature shoulder reflecting minor amplification of As1e. RFU, relative fluorescence units; T, temperature.

Because the absolute concentration of GIV cannot be guaranteed after lengthy and uncertain shipping from the United States to the Philippines, we calibrated the RT-LAMP assay by RT-qPCR, accepting that this is still a relative measure. Using minor variants of methods **[B]** and **[D]**, we determined the RT-qPCR C_t value as a function of spiked virus concentration (Fig. 6B). An RT-LAMP sensitivity of 31 and 125 copies per reaction (the lower and upper sensitivity estimates, above) corresponds to RT-qPCR C_t of ~30–32. Therefore, both methods can detect ~30–125 copies per reaction, corresponding to ~10–100 copies/μL in patient saliva, yielding a C_t of ~31 using this particular RT-qPCR method (Fig. 6C).

DISCUSSION

The intended use for the RT-LAMP test is to increase testing capacity in established clinical testing laboratories currently using NPS-derived, RNA-extracted RT-qPCR, specifically in the Philippines. The primary objective was to simplify sample collection and preanalytical processing as a means to increase testing capacity. We purposefully sought to build on the work of the wider community, particularly those in the gLAMP Consortium (this issue)—we, like many others, had not worked on LAMP before 2020. This work spans January to May 2021, with major progress made after March when we secured proteinase K. The crippling impact on productivity of extended lead times for imported reagents—measured in months not days—cannot be overemphasized.

Processing Method

Of 6 methods tested covering enzymatic (protease) and nonenzymatic, extraction-free methods, those derived from Rabe and Cepko **[D]** and Ben-Assa *et al.* **[B]** (with addition of 1 U/μL RNase inhibitor and a 1:1 dilution of saliva in **[D]**) were the most promising. Although the

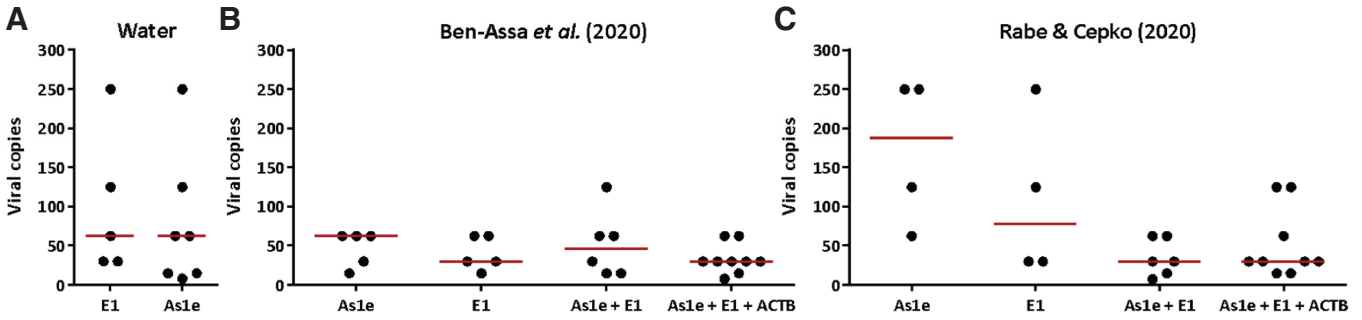

FIGURE 4

Sensitivity of As1e and E1 primer sets for amplification of SARS-CoV-2. *A*) Purified RNA diluted in water before testing. *B*, *C*) Gamma-inactivated virus spiked into healthy control saliva before being processed using the modified method by Ben-Assa *et al*. **[B]** (*B*) or the modified method by Rabe and Cepko **[D]** (*C*). Each data point represents a dose-response curve (1000–8 copies per reaction; 200–1.5 copies/μL input) identifying the lowest concentration where SARS-CoV-2 GIV was detectable within 15 min. Horizontal red lines reflect the median of each data set.

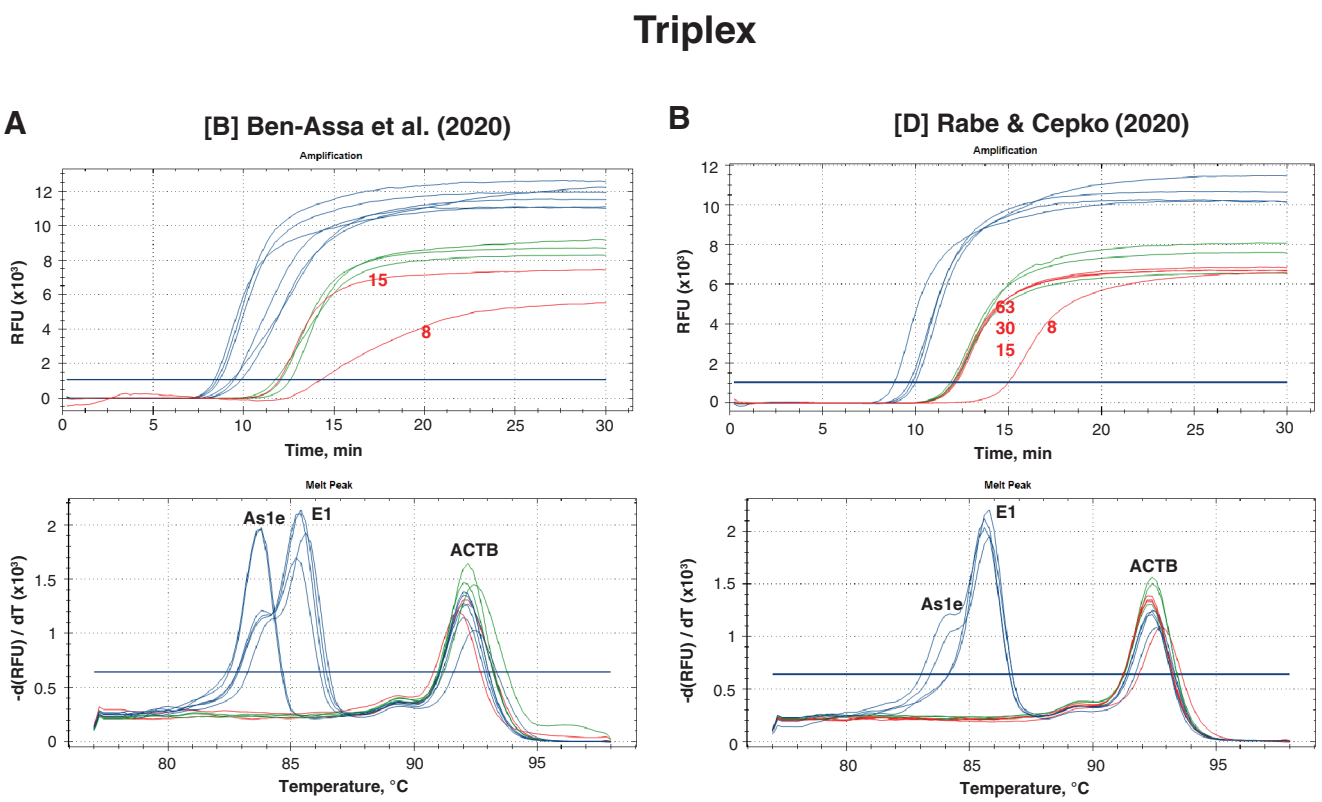

FIGURE 5

Amplification and melt curves for triplexed primer sets (As1e, E1, and ACTB). Blue (true positives): samples with at least 2 melt peaks (E1 typically dominates); green (true negatives): saliva samples that lack SARS-CoV-2 RNA; red (false negatives): only the ACTB gene is amplified despite the presence of low concentrations of viral RNA. Because all spiked saliva and NTC saliva samples contain the ACTB primer set, the NTC is represented by amplification of only ACTB (green), which is observed in all reactions, as expected of a positive internal control. RFU, relative fluorescence units; T, temperature.

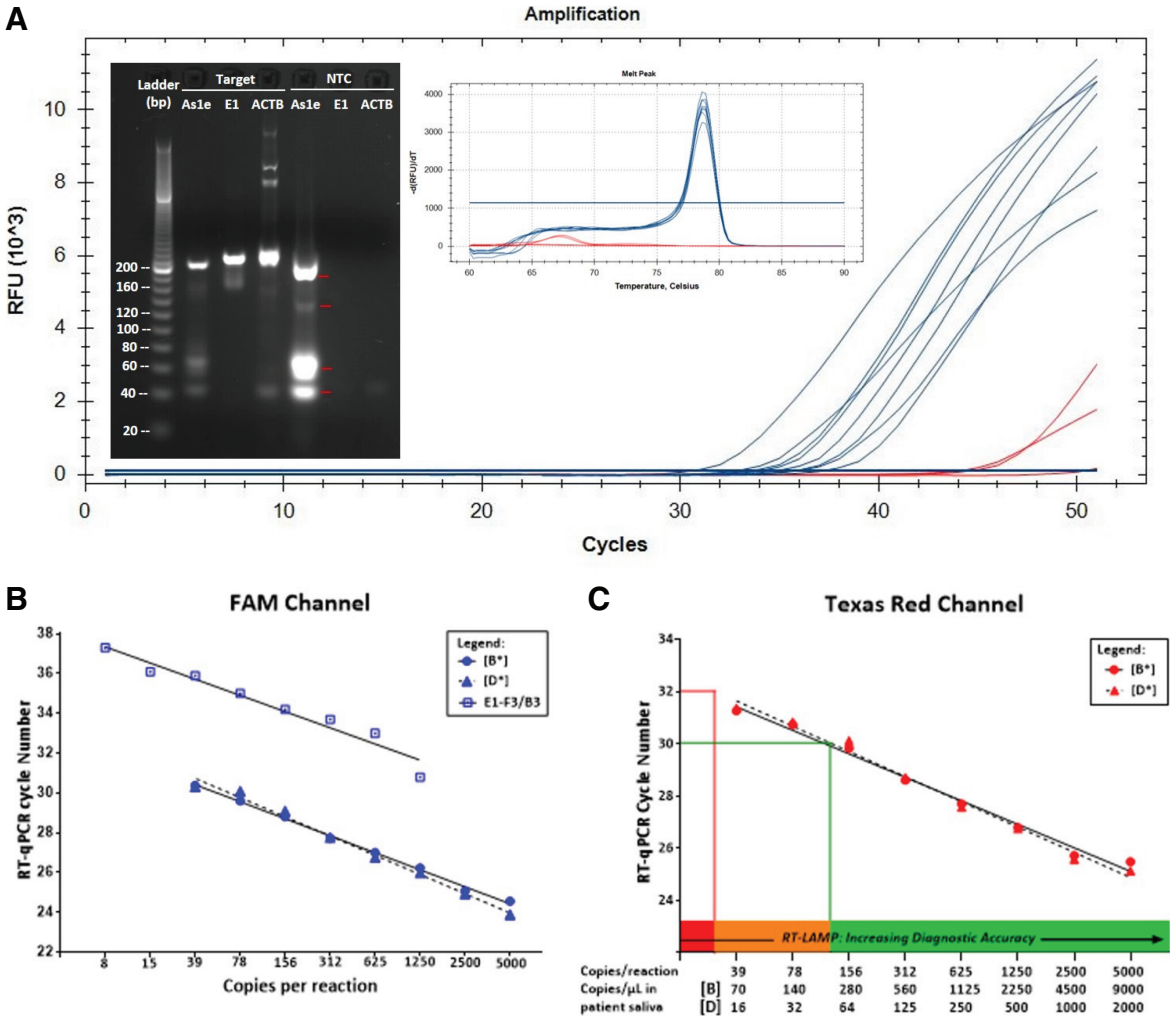

FIGURE 6

RT-qPCR amplification using conventional and LAMP F3/B3 primers. *A*) Reverse transcription PCR progress curves for amplification of 8–1000 (NTC in red) copies of purified RNA, 60°C annealing. Inset: 3% agarose gel electrophoresis; 500 copies per reaction, 51 cycles (As1e, E1) or saliva (ACTB) and melt curves for RNA amplicons (blue) and NTC (red). Nonspecific formation of 4 nonproductive amplicons with As1e is highlighted in the gel. *B*) Amplicon detection *via* intercalation (SYTO9) in saliva processed with methods **[B]** and **[D]**; open squares using less efficient E1 F3/F3 primers ($C_t = -0.81.\log_2[\text{RNA}] + 40.0$) and closed symbols using more efficient primers from the Centers for Disease Control and Prevention.[17] *C*) Amplicon detection using a hydrolysis probe; slopes of best fit lines ($C_t = -\text{m}.\log_2[\text{RNA}]+\text{c}$) from the Texas Red channel are 0.90–0.97; intercepts = 36.2–36.8. Below the *x* axis, values represent viral loads in the patient saliva, back-calculated accounting for sample processing methods. The lower limit of uncertainty is the median sensitivity (30 copies per reaction; Table 2); the upper limit is the uppermost value in the range (125 copies per reaction; Table 2). These values correspond to an RT-qPCR C_t ~30–32. bp, base pairs; RFU, relative fluorescence units.

addition of RNase inhibitors improved result reliability for a relatively small additional cost (~$0.15), it is not obligatory. With GIV spiked into healthy control saliva before processing, both methods had reliable detection down to ~30 copies per reaction in duplex (As1e + E1) or triplex (As1e + E1 + ACTB) formats. The modified method **[D]** in particular appeared to perform better in duplex than singleplex format despite E1 being predominantly amplified within the duplex. The apparent greater robustness of method **[B]** over modified method **[D]** is counterbalanced by the 4-fold greater predilution of the patient saliva before testing. Thus, when considering patient viral load, method **[D]** had the higher sensitivity. Nevertheless, both methods can detect patient viral loads above 10–100 copies/µL. These concentration ranges correspond, in our RT-qPCR assay, to a C_t ~29–32 (Fig. 6C). Using clinical

samples, methods **[B]** and **[D]** (see Anahtar *et al.*[16]) arrived at a similar cutoff value (C_t <29–30).

Primer Duplexing

We had originally intended to target the N gene set to maximize sensitivity but were surprised by the poor apparent specificity of both the Color N and N2 primer sets given their widespread use in the research community.[3] We speculate that our particular batches of primer had impurities that led to mispriming. Development of the duplex (As1e + E1) is primarily intended to increase genomic coverage, reducing reliance on any one primer set, rather than as a means to increase sensitivity. Indeed, any effect of duplexing on assay sensitivity in the enzymatic method **[B]** was marginal. In contrast, the nonenzymatic method **[D]** seemingly performed better in duplex *vs.* singleplex format with improvements in both absolute sensitivity and in robustness between experiments (Fig. 4; Table 2). We might speculate that this reflects the suboptimal and/or more variable release of RNA in the absence of proteinase K and is partly compensated for by the presence of 2 primer sets. Proteinase K is known to improve extraction of nucleic acids from clinical samples by digesting RNAses, preventing degradation of SARS-CoV-2 RNA and/or removing amplification inhibitors.[15] Although we sought to use "clear drooled saliva" to aid processing,[6] proteinase K may assist in homogenization of any samples that contain more viscous secretions from the nasopharynx or airway.

Internal Control

LAMP assays with an internal control (often a human housekeeping gene) usually have a sequencing[29] or multicolor[23] readout. Here we too included an ACTB primer set as a positive amplification control, which, after suitable desensitization, did not crucially affect sensitivity for the As1e and E1 SARS-CoV-2 genes. Because this single-color method does not spectroscopically distinguish the ACTB, As1e, and E1 amplicons, we instead distinguish them thermodynamically *via* melt curves (Rolando *et al.*[30]; Fig. 5). Necessarily this requires access to instruments with this capability; our intended use-case is in secondary and tertiary clinics that have such RT-qPCR instruments. Other cost-effective incubator-readers with melt capability are available[6] that can extend use of the triplex assay beyond a central laboratory.

The internal control removes the need to run separate external controls, a feature that becomes more attractive as the batch run size gets smaller. It increases per-well confidence when using saliva, a matrix that is inherently complex and can be challenging to work with when collected from different donors (see Tan *et al.*[6] for a short but thorough review). To qualify as an extraction control, we expected ACTB amplification only in processed (extracted) saliva but observed amplification in the absence of processing (data not shown), presumably because sufficient RNA is released during the 65°C incubation step. Thus, ACTB formally serves as an amplification control, not an extraction control. We also acknowledge that our study used saliva pooled from individuals all with similar demographic profiles and lifestyles (university researchers)—we foresee increased variability when the test is applied to the wider patient population with different lifestyles and diets. Finally, we highlight that this preclinical work was completed with only one lot of controls (RNA and GIV) from BEI Resources and that these reagents have an unknown transport and import provenance from the United States through Customs in the Philippines.

Nonspecific Amplification

Although we initially encountered widespread "nontemplate" amplification, it became apparent that this was primarily due to cross-contamination rather than mispriming.[31] We attribute improvements in controlling cross-contamination partly to the dUTP/UDG system (which had minimal impact on sensitivity/time to positive values; data not shown) and partly to improved and more rigorous working practices. An important aspect of any deployment of LAMP must be to highlight to end users the key requirements and pitfalls when working with LAMP, particularly if, as in many low- and middle-income countries, the level of training and infrastructure for healthcare workers can be highly variable at best. Despite initial problems and some notable exceptions (Color N and N2 primer sets), we generally observed minimal off-target amplification when using LAMP with saliva (*e.g.*, Fig. 5)—and when nonspecification amplification occurs it can be readily distinguished *via* melt analysis.

Clinical Utility

This is a pilot study using contrived samples. Yet, we might reasonably predict the assay to detect viral loads of 100 copies/μL and above with confidence (Fig. 6C; RT-qPCR C_t ≤30). The region of uncertainty (10–100 copies/μL patient saliva) encompasses sensitivities described by others in the literature (median, 75 copies/μL; mean, 90; range, 10–200; unpublished results). Indeed, comparable results to ours were obtained by Ben-Assa *et al.* and Rabe and Cepko.

Although the LAMP test is 10–100-fold less *analytically* sensitive than PCR, the *clinical* question is whether the LAMP limit of detection is fit for purpose.[19,20,32–34] "Adequate sensitivity" depends on one's circumstances and on the intended users and use-case. In the Philippines

and other low- and middle-income countries, RT-qPCR testing is neither economically nor geographically accessible to many. Whether due to the lack of local hospital and health care infrastructure or to the high cost of testing relative to income, many are isolated from affordable diagnostics.

Here as elsewhere, test utility must consider accessibility factors over and above sensitivity and specificity, important as these undoubtedly are (see Land *et al.*[35]). *The sensitivity and specificity of a test that for logistical, economic, or geographical reasons never reaches the patient are operationally zero*. Clinical testing is underway to determine whether the RT-LAMP assay can be used to meet the local demand for simpler and cheaper SARS-CoV-2 testing that is accessible to all.

ACKNOWLEDGMENTS

The authors are very grateful to New England Biolabs (Ipswich, MA, USA) and New England Biolabs Singapore for helping make this project possible, particularly to Sheila Mendoza, Dr. Nathan Tanner, and Dr. Eileen Dimalanta—*maraming salamat po*. The authors acknowledge the generous gift of control RNA from BEI Resources, of HEK cells from Dr. R. Garcia (University of the Philippines), useful discussions with Dr. R. Guerrero, and the assistance of many members of the gLAMP Consortium. The authors thank the Reviewer for their very helpful comments and suggestions for improvement. K.J.M.M. dedicates this paper to the memories of Dr. Ian R. Moore, University of Oxford (1964–2018), and Lesley J. Moore (1964–2020). This study was funded *via* government grants-in-aid (SWIFTeR and ADDS projects) from the Philippines Council for Health Research and Development in the Department of Science Technology (PCHRD-DOST).

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