Original Research

Improved protocol for *Bst* polymerase and reverse transcriptase production and application to a point-of-care diagnostics system

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Impact Statement

Bst polymerase and reverse transcriptase were obtained in high yield and purity and used in reverse transcription loop-mediated isothermal amplification (RT-LAMP) applied to the development of a one-step reaction mix to detect SARS-CoV-2, using a portable point-of-care device. The application of the system can be expanded to other infectious agents, decreasing the response time, and expanding diagnostic tests in remote areas.

Abstract

The pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has raised awareness in the scientific community about the importance of being prepared for sanitary emergencies. Many measures implemented during the COVID pandemic are now being expanded to other applications. In the field of molecular and immunological diagnostics, the need to massively test the population worldwide resulted in the application of a variety of methods to detect viral infection. Besides gold standard reverse transcription quantitative polymerase chain reaction (RT-qPCR), the use of reverse transcription loop-mediated isothermal amplification (RT-LAMP) arose as an alternative and sensitive method to amplify and detect viral genetic material. We have used openly available protocols and have improved the protein production of RT-LAMP enzymes *Bst* polymerase and *HIV*-reverse transcriptase. To optimize enzyme production, we tested different protein tags, and we shortened the protein purification protocol, resulting in reduced processing time and handling of the enzymes and, thus, preserved the protein activity with high purity. The enzymes showed significant stability at 4 °C and 25 °C, over 60 days, and were highly reliable when used as a one-step RT-LAMP reaction in a portable point-of-care device with clinical samples. The enzymes and the reaction setup can be further expanded to detect other infectious diseases agents.

Keywords: *Bst* polymerase, reverse transcriptase, RT-LAMP, SARS-CoV-2, RNA virus detection, point-of-care

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Introduction

When the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic was officially declared in March 2020,[1] the health authorities and the scientific community established guidelines pointing to the importance of massive testing of the population. The identification of positive cases and their isolation were fundamental to reduce the speed of viral transmission, allowing time for the definition of treatment options and for vaccine development.[2,3] The necessity of testing was met with an extraordinarily fast response worldwide, and efficient technologies were used for diagnosis. However, the race to achieve test coverage resulted in a shortage of reagents, equipment, and trained personnel in many world regions.[4,5]

Since the beginning of the pandemic, the RT-qPCR (reverse transcription quantitative polymerase chain reaction) test has been proposed by the World Health Organization (WHO) as the gold standard method for the detection of SARS-CoV-2 virus, due to its well-known sensitivity and the possibility of early detection of viral particles.[6,7] However, with the shortage of reagents, time-consuming execution of the method,

expensive equipment requirements, and low usability and accessibility, other nucleic acid-based diagnosis techniques started to be developed.[8,9]

The loop-mediated isothermal amplification (LAMP) reaction[10] arose as an alternative method in this scenario because it has equivalent sensitivity to quantitative polymerase chain reaction (qPCR),[11,12] is more easily executed, and can be coupled with a reverse transcription reaction (RT-LAMP) for RNA detection.[13,14] This technique dispenses the need for a thermocycler and nucleic acid extraction, enabling the use of minimally processed samples.[9,10,15,16]

RT-LAMP requires a compatible reverse transcriptase and a thermostable, non-exonuclease DNA polymerase capable of strand displacement.[13,14] The reverse transcriptases used in RT-LAMP are the commonly available, such as *M-MLV* RT (*Moloney Murine* leukemia virus reverse transcriptase),[17] *AMV* RT (*Avian Myeloblastosis* virus reverse transcriptase),[17] Thermophilic RT (from *Thermus thermophiles* reverse transcriptase),[18] and *HIV* RT (*Human Immunodeficiency* virus reverse transcriptase).[19] Concerning DNA polymerases, the mostly used for RT-LAMP is the *Bst* DNA Polymerase (*Bacillus stearothermophilus* DNA polymerase)[10] which possess an enhanced strand displacement capability, allowing the enzyme to efficiently displace and replicate DNA strands in isothermal setups. The *Bst*-LF (large fragment) used by us and by others represents the portion of the enzyme containing $5'\rightarrow3'$ polymerase activity but lacking $5'\rightarrow3'$ exonuclease activity. Among the commercial enzymes, some are customized by the introduction of small modifications, and available from companies as NEB,[20] Lucigen,[21] OptiGene,[22] TakaraBio,[23] and ThermoScientific.[24] During the reaction, primers recognize 6 to 8 distinct regions of the target DNA and promote the formation of loops in the synthesized cDNA. These loop structures facilitate the subsequent rounds of amplification, as the loops can be extended, adding new primer binding sites and increasing sensibility.[25] Another advantage of the method is that amplified DNA can be detected by several different techniques, such as agarose gel,[10] turbidimetry,[26] pH change detection with pH indicators (colorimetric),[27] through the use of intercalating DNA fluorophores (fluorometric),[10] or oligonucleotide strand exchange probes.[28,29] The possibility of using unprocessed samples and the simplicity of a single-step isothermal reaction, without the need for complex equipment, contributed to the fast dissemination of the LAMP-based tests in comparison to PCR.

Regardless of the chosen diagnostic method, the establishment of reliable protocols to produce enzymes with high yield and activity that are not covered by patents is essential to avoid the worldwide reagent shortage problems that happened during the COVID-19 pandemic.[30–32] This learned lesson may be crucial to deal with future threats of new pandemics, such as other respiratory and tropical diseases caused by infectious agents.[33,34]

Another important lesson learned from the pandemic is the need to decentralize test performance, allowing for both faster diagnosis and access to remotely populated areas. In this context, clinical point-of-care (PoC) molecular systems provide an efficient solution to these problems, allowing for reliable diagnosis and prompt report of positive cases to the health authorities. The PoC systems are mostly defined by the reach of simple, portable devices that allow sample collection everywhere and the remote delivery of results by specialists.[35,36]

In this work, we established an optimized protocol for high-yield production and quality control of enzymes needed for RT-LAMP, which were not covered by patents. Both enzymes, *Bst* DNA Polymerase and Reverse Transcriptase, showed high stability even when kept in non-refrigerated conditions for a limited time, which is an advantage in the case of transport needs during pandemics. These enzymes were used to develop a SARS-CoV-2 diagnosis test based on the RT-LAMP method and applied to a colorimetric PoC platform that uses artificial intelligence (AI) to analyze the results.[37]

Materials and methods

Reagents and equipment

Luna Universal qPCR Master Mix, WarmStart® RTx Reverse Transcriptase, *Bst* 2.0 WarmStart® DNA Polymerase, and Diluent A were acquired from New England Biolabs (NEB). Betaine, magnesium sulfate, Tween-20, Phenol red, potassium chloride, potassium hydroxide, Phenylmethanesulfonyl fluoride (PMSF), Triton™ X-100, and Molecular biology-grade Glycerol were obtained from Sigma-Aldrich. Isopropyl 1-thio-D-galactopyranoside (IPTG), Phenylmethylsulfonyl fluoride (PMS), Guanidine-HCl, HEPES and Tris (2-carboxyethyl) phosphine hydrochloride 98% (TCEP) were acquired from Apollo Scientific. Deoxyuridine triphosphate (dUTP), Uracyl DNA glycosylase (UDG), EvaGreen, and ROX were acquired from Cellco. The deoxynucleotide (dNTP) mix was acquired from Promega. SuperScript™ II, SuperScript™ III Reverse Transcriptase, Tris-EDTA (TE) buffer, and UltraPure DNase/RNase-Free Distilled Water were obtained from ThermoFisher Scientific. Potassium chloride and bromothymol blue were acquired from Química Moderna and Êxodo Científica, respectively. Sodium chloride and glycerol were obtained from Synth. Ammonium sulfate and EDTA disodium salt were obtained from Merck Millipore. Tris base was purchased from Melford. Molecular biology grade DTT (dithiothreitol) and imidazole were acquired from Fisher Bioreagents and Chem-Impex, respectively. Reagents obtained from different suppliers are indicated in the main text. Regular PCR and reverse transcription reactions were performed in the TAdvanced PCR system (Biometra). The colorimetric LAMP and RT-LAMP reactions were executed in the Hilab Molecular system (Hi Technologies) and the fluorescent LAMP/RT-LAMP, and quantitative PCR were executed in the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems).

Cloning of CQMED GO-RT, HIV-RT, and Bst-LF DNA polymerase

Synthetic plasmid for CQMED *GO*-RT (pET28(a)-GO-RT) was obtained from GenOne (Brazil). The cDNA templates of *HIV* reverse transcriptase (Addgene plasmids #153311 and #159149) and *Bst*-LF DNA polymerase (Addgene plasmid #159148)[19] were amplified by PCR and cloned into the pET28-derived plasmids pNic28-Bsa4, pNic-CTHO and pNic-Zb[38] using ligation-independent cloning (LIC; Supplemental

Table S1).[39] Constructs were verified by Sanger DNA sequencing. *Escherichia coli* Mach-1 cells (T1 phage-resistant, Invitrogen) were used for plasmid transformation.

Protein expression and purification

For protein production, the chloramphenicol-resistant *E. coli* BL21 derivative strain Rosetta™ 2 (Merck Millipore) was used. To assess protein solubility, a small-scale expression and purification test of 1 mL cultures was performed, as described elsewhere.[38] BL21(DE3)-Rosetta 2 cells harboring the selected plasmids were grown in TB medium (supplemented with 50 $\mu g/ml^{-1}$ kanamycin, 35 $\mu g/ml^{-1}$ chloramphenicol) under agitation (140 r/min) at 37 °C until OD_{600} reached approximately 2.0. The cell culture was then cooled to 18 °C for 30 min, prior to protein expression induction by the addition of 0.2 mM isopropyl 1-thio-D-galactopyranoside (IPTG). The culture was kept overnight at 18 °C under shaking for 16–18 h. Cells were harvested by centrifugation (15 minutes, 6000*g*, at 4 °C) and the pellets suspended in 2× lysis buffer (1 mL per gram of cells—100 mM HEPES pH 7.4, 1 M NaCl, 20% glycerol, 20 mM imidazole, and 1 mM TCEP) supplemented with 1 mM PMSF. Cellular suspensions were flash-frozen in liquid nitrogen and stored at −80 °C until use. For purification, the cellular stocks were thawed and sonicated on ice for 4 min (5 s on, 10 s off; amplitude = 35%) using a Sonics Vibra Cell VCX750 ultrasonic cell disruptor (Sonics). The samples were centrifuged (45 min, 56,000*g*, at 4 °C), and the clarified lysates were applied on a 5 mL HisTrap™ FF Crude column (Cytiva) connected to an ÄKTA pure FPLC system (Cytiva) for immobilized metal affinity chromatography. The proteins were eluted with 300 mM imidazole in lysis buffer (50 mM HEPES-NaOH pH 7.4, 0.5 M NaCl, 10% glycerol, and 0.5 mM TCEP). Removal of the 6×His-tag was performed using recombinant TEV protease while dialyzing against appropriate digestion buffer (See Supplemental Table S2) in a dialysis bag (SnakeSkin™ Dialysis Tubing 10 kDa MWCO—Life Technologies) for 16–18 hours at 4 °C. The protein solutions were further purified using reverse affinity chromatography on Ni^{2+}-Chelating Sepharose (GE Lifesciences) columns, with the untagged proteins collected in the flow-through fraction or eluted with an imidazole step gradient (up to 60 mM) in the same buffer. The fractions of interest were pooled and concentrated to 3 mg/mL or more, prior to being submitted to a second dialysis with 2× storage buffer (Supplemental Table S2), in a 10 kDa MWCO dialysis bag overnight at 4 °C. Glycerol, DTT, and EDTA were added to the dialyzed proteins to achieve the desired final stock enzyme composition. Enzymes were stored at −30 °C until use. Since we are obtaining enzymes for activity on nucleic acid, all solutions were prepared with sterile deionized DEPC-treated water.

Qualitative PCR to evaluate *E. coli* gDNA in protein preparations

Quantitative PCR was performed in 10 µL reactions using Luna Universal qPCR Master Mix with QuantStudio 6. Reactions were run with a final primer concentration of 0.25 µM each at 95 °C for 60 seconds, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, with a final melt curve step (60–95 °C).

Two-step RT-qPCR to evaluate reverse transcription activity of the enzymes GO-RT, HIV-RT, and Bst-LF

Many of the naturally occurring polymerases also present a residual reverse transcription activity and could eventually be used as a dual RT-polymerase enzyme, although the activity varies greatly. We evaluated the RT activity in the produced enzymes by a reverse transcription reaction of 25 µL, prepared using different concentrations of CQMED enzymes or commercial equivalents: WarmStart RTx Reverse Transcriptase, SuperScript II, or SuperScript III Reverse Transcriptase. Reactions were carried out in Isothermal buffer (20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% Tween-20) at designated temperatures with 0.1 µM GAPDH reverse primer and 0.5 mM dNTP mix. A 10-min reaction time was used unless specified otherwise. Mg^{2+} supplementation requirement was also evaluated. To test the RT activity, we used HeLa cells' total RNA, extracted using PureLink™ RNA Mini Kit (Thermo Fisher Scientific), treated with DNAse (TURBO DNA-*free*™ kit—Invitrogen), and used as a template in all reactions. For the qPCR step, 1 µL of cDNA from RT reactions was used as a template with forward and reverse primers (Supplemental Table S1) targeting the human GAPDH gene (GenBank M33197.1), applying the cycling conditions described above.

Polymerase activity and enzyme stability of the Bst-LF by LAMP

LAMP reactions of 10 µL were prepared with our enzyme, CQMED *Bst*-LF, or the commercial equivalent, *Bst* 2.0 WarmStart DNA Polymerase, in the Isothermal buffer. The reactions were supplemented with 6 mM $MgSO_4$ and contained 1.4 mM dNTP mix, 0.7 mM dUTP, 0.005 U/µL UDG, 400 mM Betaine, 40 mM Guanidine-HCl pH 8.0, 0.5× EvaGreen, 0.5× ROX Dye, and primer mix targeting the N2 gene of SARS-CoV-2 in a final concentration of 1.6 µM for FIP/BIP, 0.2 µM for F3/B3 and, 0.4 µM for the loop primers (LF/LB) (Supplemental Table S1). pUC57 plasmid containing the N2 gene sequence cloned in the EcoRV site (GenScript) was used as the positive control.[37] Reactions were run at 67 °C for 60 cycles of 60 s, unless specified otherwise, with data collected on the SYBR channel at the end of each cycle.

Colorimetric LAMP and RT-LAMP performed using the Hilab Molecular equipment

In the Hilab Molecular device,[37] 20 µL colorimetric LAMP/RT-LAMP reactions were prepared in solution with 50 mM KCl, 8 mM $MgSO_4$, 0.1% (v/v) Tween-20, 0.2 µL of the corresponding DNA polymerase component, 0.4 µL of the reverse transcriptase element, and 4 µL of sample solution (1 mM Tris-EDTA, 0.1% (v/v) Tween-20, 500 µM Phenol Red). The reactions also contained 100 µM Bromothymol Blue, 0.3 mM KOH, 1.4 mM dNTP Mix (1.4 mM dATP, dCTP, dGTP, and 0.7 mM dTTP, dUTP—New England Biolabs), 0.005 U/µL UDG (New England Biolabs), 400 mM Betaine, 40 mM Guanidine-HCl (Sigma-Aldrich), and primers targeting SARS-CoV-2 genes E1 and N2 (1.6 µM FIP/BIP, 0.2 µM

F3/B3, 0.4 µM LF/LB of each target—IDT) (Supplemental Table S1). Reactions had 300 µM (CQMED) to 500 µM (NEB) of Tris from the enzymes' buffer carryover.

For CQMED *Bst*-LF optimal concentration determination, LAMP reactions were set up with 10,000 copies per reaction of pUC17 plasmids with the SARS-CoV-2 regions of interest (N2 and E1) diluted in the sample solution. NEB *Bst* 2.0 WarmStart enzyme was used as a reference in the previously optimized concentration of 0.4 U/µL,[40] and CQMED *Bst*-LF concentration varied from 4 to 12 ng/µL, both diluted in Diluent A to 100×, so the concentration of the enzyme buffer remains the same in all reactions.

For the Reverse transcriptase activity assessment in RT-LAMP assays, CQMED *Bst*-LF at 12 ng/µL was used as the DNA polymerase element in all reactions, combined with either WarmStart RTx Reverse Transcriptase at 0.3 U/µL final concentration or CQMED *GO*-RT/*HIV*-RT enzymes varying from 1 to 8 ng/µL. All RTs were diluted to 50× with Diluent A. About 10,000 copies per reaction of Twist Biosciences SARS-CoV-2 RNA was used as a positive control. Reactions were run for 1800 or 3600 s at 65 °C. The colorimetric reactions are interpreted in terms of the amplification initiation time (Cy0) and the maximum signal (delta—N_{max}). Cy0 is a parameter equivalent to the qPCR Ct that accounts for the initial time of an amplification,[41] while N_{max} is described as the delta achieved by the reaction. In our experiments, we defined that reactions with N_{max} below 25 and/or Cy0 above 1200 s are considered negative.

Stability assay in adverse temperature conditions

Liquid aliquots of CQMED enzymes were stored at −20 °C, 4 °C, and at room temperature (22–25 °C), and the activities were assessed after different periods of time, by LAMP (for DNAPol) and RT-qPCR (for RTs), as described above. The −20 °C storage condition was used as a control.

Investigation of RNase/DNase activity assay

Nuclease residual activities of CQMED enzymes were determined using the commercial RNase + DNase Detection Kit (Jena Bioscience), based on the measurement of the degradation of fluorescent RNA/DNA probes. Reactions were set up according to the manufacturer's instructions and compared to standard controls containing RNase A and DNase I. CQMED enzymes were diluted to a final glycerol concentration of 2%. Reactions of 20 µL were prepared with 10 µL of the sample (CQMED enzymes, standards, or PCR-grade water) and 10 µL of Detection Master Mix. Samples were run in a QuantStudio 6 at 67 °C for 30 min (one cycle per minute), and data were collected on FAM and VIC channels at the end of each cycle.

Investigation of contaminating bacterial gDNA

To prevent the influence of our polymerases on this DNA amplification-based quantitative assay, we employed a proteolytic digestion protocol using proteinase K (PK), adapted from a previous report.[42] For each 50 µL reaction, 0.2 mg/mL of PK was used. Using a TAdvanced Thermal cycler (Biometra), samples were incubated at 55 °C for 2 h, followed by PK inactivation at 90 °C for 10 min. Quantitative PCR was performed, as described above, using forward and reverse primers targeting the 16S gene of *E. coli* (Supplemental Table S1), designed using NCBI Primer-BLAST software and based on the conserved 16S ribosomal subunit sequence (GenBank J01859.1). CQMED enzymes were tested at a final concentration of 12 ng/µL. A standard curve of 10-fold serial dilutions was prepared using gDNA extracted from DH10Bac strain (Thermo Fisher Scientific), using commercial miniprep solutions (Cell Resuspension Solution, Cell Lysis Solution, and Neutralization Solution-Merck Millipore).

Polymerase activity assay of Bst-LF for relative unit determination

To determine the amount of CQMED *Bst*-LF DNA polymerase that corresponds to 1 Unit (U), we applied the EvaEZ™ Fluorometric Polymerase Activity Assay Kit (Biotium) in a QuantStudio 6 using the manufacturer's protocol, based on the number of nucleotides incorporated during the reaction. The assay was performed at 67 °C for 60 min (one cycle per minute), and data were collected on the FAM channel. Saturating concentrations of CQMED *Bst*-LF were used to determine the maximum fluorescence change (ΔF_{max}) of the experiment, which corresponds to the consumption of 270 pmol of nucleotides. The fluorescence changes (ΔF) calculated from the initial activity rates (slope) of non-saturating enzyme reactions were then used to determine the number of consumed nucleotides through the formula ($\Delta F/\Delta F_{max}$*270 pmole) in 60 min. We considered 1 U as the amount of CQMED *Bst*-LF necessary to incorporate 25 nmol of dNTPs in 60 min at 67 °C in a Tris-buffered system containing 2.5 mM Mg^{2+}.

Tests with clinical samples

The COVID-positive nasopharyngeal swab samples used in our analysis were previously validated in another study.[37] These samples were homogenized in a 500 µL solution containing 0.1% (v/v) Tween-20 and 1 mM Tris-EDTA buffer, and subsequently stored at temperatures of −70 °C and −30 °C. We tested these samples using the optimized RT-LAMP settings with both NEB's and CQMED enzymes.

To ensure the comparison between enzymes was conducted on non-degraded samples, we included only the 28 samples that were confirmed as positive in a revalidation RT-PCR and showed positivity in at least one of the RT-LAMP reactions for analysis. The samples were categorized based on their previously obtained Cts in an RT-qPCR assay, including Negative, 15–20, 20–25, 25–30, and above 30. The reactions were performed using the Colorimetric RT-LAMP reaction, as previously described, and were run in the Hilab Molecular.

The RT-qPCR assay was performed with primers designed to target the N1 and E1 genes of SARS-CoV-2. Clinical samples underwent cDNA synthesis using a two-step RT-qPCR protocol from New England Biolabs (1 µL of sample, 1× Isothermal Amplification Buffer, 0.5 mM dNTP, 6 µM reverse primers for N1 and E1[43] genes, 0.25 µL WarmStart RTx

Reverse Transcriptase in a final reaction volume of 10 µL). Following cDNA synthesis, 1 µL was utilized as a template for the qPCR analysis (0.1 µM forward and reverse primers for N1 and E1 genes, 0.038 U/µL Taq enzyme, 1× Taq buffer, 2 mM MgCl2, 0.2 mM dNTP, 5 µM Syto 9, and 1× ROX reference dye to a total volume of 10 µL) using QuantStudio 3.

Statistical analysis

Analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to perform comparisons between groups. Simple linear regression was used to predict dependent variables and enzymatic efficiencies. All analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, California USA) and results are expressed as means (SD). A statistically significant difference is expressed by p value $< .05$ and represented by (*). Unless otherwise stated, all experiments were executed as two independent experiments with three technical replicates each.

Results

Optimized expression and purification steps of enzyme production resulted in products with high purity and yield

Using LIC, the corresponding *Bst*-LF and *HIV*-RT coding sequences were transferred into three different expression vectors, which differ in tag type (6xHis-tag or 6xHis-Zb-tag) and position (N- or C-terminal), to allow the identification of the most efficient expression clones.

A small-scale expression (1 mL culture) and purification test was carried out using the BL21(DE3)-Rosetta 2 cells containing the different constructs (Figure 1(A)). The additional *GO*-RT enzyme was expressed from a commercial pET28a plasmid (N-terminal 6xHis-tag, "CQMED *GO*-RT") using the same protocols. All generated clones induced good expression levels of soluble proteins within the expected molecular weight. Based on the highest level of protein expression, we decided to proceed with N-terminal His-tagged constructs, now called CQMED *HIV*-RT and CQMED *Bst*-LF for large-scale expression and purification (Figure 1(C) and (D)). Initially, to assess the protocol feasibility and protein production yield, the selected clones were submitted to a large-scale expression and purification round from 1.5 L cultures, using the standard pipeline from our laboratory.[44] This pipeline relays in a three-step purification protocol (1—IMAC, 2—TEV digestion with reverse IMAC, and 3—Size exclusion chromatography), including an additional, final dialysis using 50% glycerol-containing buffer (v/v), prior to storage. This protocol was modified (Figure 1(B)), aiming to optimize the enzyme production to obtain increased protein yield per round and reduce the processing time and handling to preserve the enzyme activity and purity. The size exclusion chromatography (Gel filtration) step was removed because we observed that it did not improve protein purity and contributed to a yield reduction. We also adjusted the TEV digestion buffer for each enzyme and added another dialysis step after the reverse IMAC to favor mild buffer changes throughout the purification process up to storage. Buffer compositions are listed in Supplemental Table S2. Figure 1(C) and (D), and Supplemental Figure S1 refers to these purification steps applied to the production of CQMED *Bst*-LF, CQMED *HIV*-RT, and CQMED *GO*-RT, respectively. The masses of the purified proteins were confirmed by liquid chromatography coupled to intact mass spectrometry (LC/MS) (Supplemental Figure S2).

With this final protocol, we were able to obtain enough enzymes for up to 500,000 RT-LAMP tests per liter of protein culture, considering the RT-LAMP setting discussed later in this text. The published protocol applied by Kellner *et al.*,[19] to produce *HIV*-RT, reported a yield of 55,000 reactions per liter, according to the applied RT-LAMP protocol.

The in-house enzymes showed comparable activities to commercially equivalent enzymes in RT-qPCR and RT-LAMP

After establishing a protocol to obtain high yields of all three enzymes, we aimed to evaluate their activity in comparison to the commercially available equivalents. For this purpose, the DNA polymerase and strand displacement activities of CQMED *Bst*-LF were tested in LAMP reactions. The *in-house* produced *Bst*-LF showed polymerase activity in the LAMP reaction, specifically amplifying the target gene in less than 15 min of reaction, even in the presence of the lowest template concentration tested of 0.05 ng (Figure 2(A)). The CQMED *GO*-RT reverse transcriptase activity was assayed and compared to the commercially equivalent enzyme SuperScript II in its optimal temperature (50 °C) and buffering conditions, in a two-step RT-qPCR protocol. The obtained results showed that *GO*-RT has very similar indirect transcription activity efficiency, compared to the reference enzyme for all tested concentrations (Figure 2(B)), as shown by the superimposed regression lines. The CQMED *HIV*-RT exhibits reverse transcriptase activity efficiency for typical cDNA synthesis nearly identical to SuperScript III, CQMED *GO*-RT, and WarmStart RTx (NEB) at 55 °C in Isothermal buffer, at the tested concentrations (4 ng/µL for CQMED in-house produced enzymes, 0.23 U/µL of WarmStart RTx and 10 U/µL of SuperScript III) (Figure 2(C)).

The *Bacillus stearothermophilus* DNA polymerase has an intrinsic reverse transcriptase activity, like many of the known polymerases,[45,46] which was evaluated alongside the RT enzymes in reactions supplemented to 8 mM $MgSO_4$, as in RT-LAMP setups, for the preservation of the polymerase activity (Figure 2(C)). The activity of all three enzymes was then tested at 63 °C and 67 °C, with different Mg^{2+} supplementation because these temperatures are more appropriate for LAMP isothermal amplification. In LAMP ideal temperatures (Figure 2(E) and (F)), CQMED *GO*-RT and *HIV*-RT maintain their activities, with no significant influence from $MgSO_4$ supplementation, while CQMED *Bst*-LF activity, as expected, decreases in its absence, as shown by the higher cycle threshold value (Figure 2(D)). Although these results seem to indicate the possibility of using *Bst*-LF as a single LAMP enzyme, it has been previously reported that *Bst*-LF alone does not work efficiently in the RT-LAMP reaction.[19]

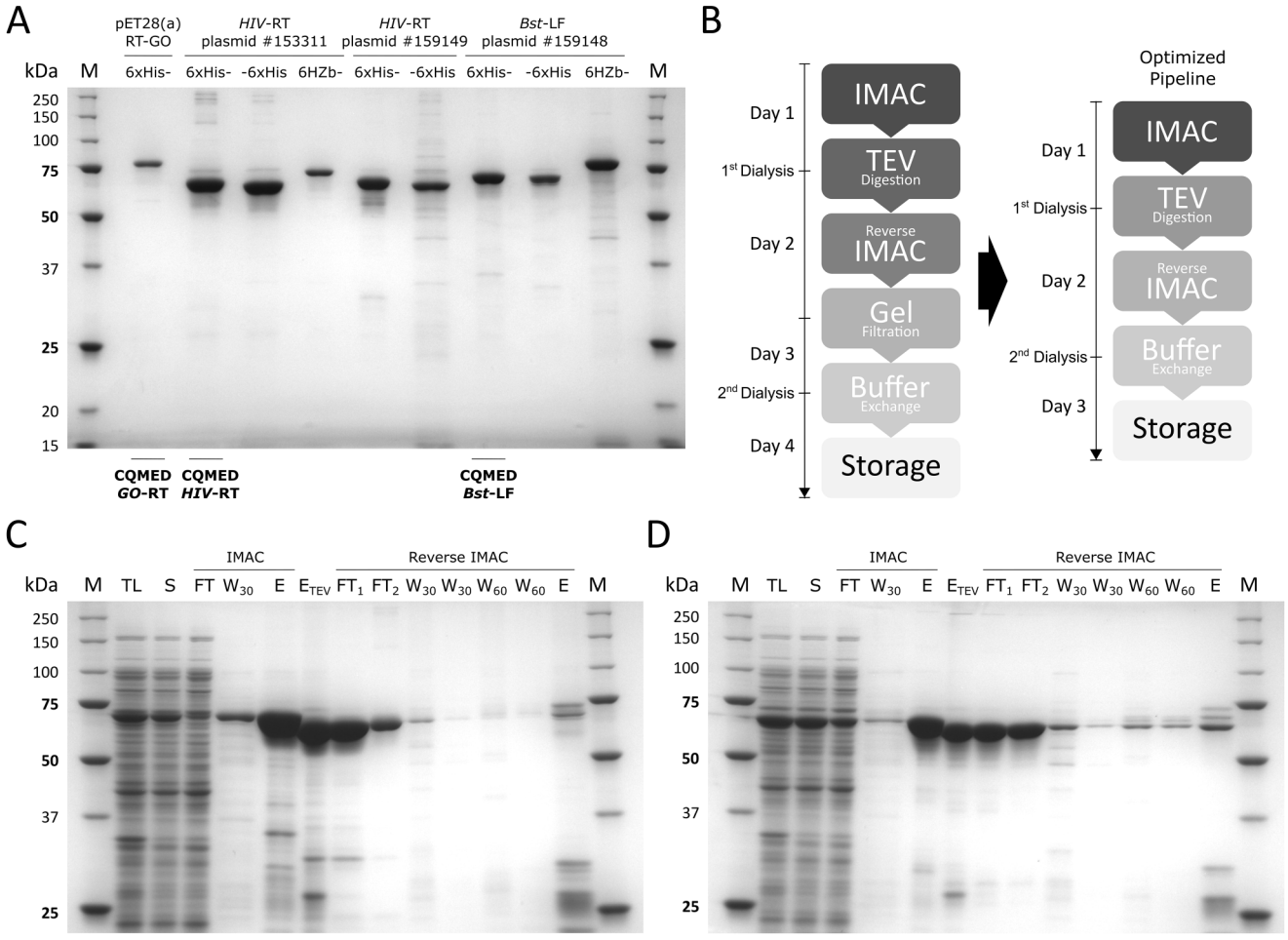

Figure 1. Protein constructs generated and steps of improvement during protein expression and purification. (A) Eluted fractions of a small-scale expression test: BL21(DE3)-Rosetta 2 cells containing different vectors harboring the target enzyme sequences were cultured in a 1 mL scale and the protein expression was induced by the addition of IPTG overnight. The supernatants from the lysed cells were submitted to a purification test with Ni^{2+}-Sepharose and the proteins of interest were eluted with imidazole. 12% SDS-PAGE Gel. 6xHis-, N-terminal His-tag; -6xHis, C-terminal His-tag; 6HZb-, N-terminal His- and Zb-tag. (B) Purification pipeline used for the production of CQMED enzymes, adapted to improve the yield in a reduced time. (C) CQMED *Bst*-LF and (D) CQMED *HIV*-RT IMAC and reverse IMAC purification steps. 12% SDS-PAGE. M, Molecular weight marker (Precision Plus Protein™ Unstained Standards (Bio-Rad); TL, total lysate; S, supernatant; FT, flow-through; W_{30}, wash with 30 mM imidazole; W_{60}, wash with 60 mM imidazole; E, elution with 300 mM imidazole; E_{TEV}, elution fraction incubated with TEV protease.

Next, we tested the produced enzymes in LAMP reactions designed to detect the SARS-CoV-2 N2 and E1 genes, using the portable Hilab Molecular equipment.[37] The titration of CQMED *Bst*-LF revealed a good correlation between the initial time of amplification (Cy0) and maximum signal (N_{max}) values obtained for the concentrations of 8–12 ng/µL of CQMED *Bst*-LF and *Bst* 2.0 WarmStart at 0.4 U/µL (Figure 2(G)), with smaller Cy0 observed at the highest *Bst*-LF tested concentration. CQMED *GO*- and *HIV*-RT, combined with 12 ng/µL of CQMED *Bst*-LF, were titrated in RT-LAMP reactions containing SARS-CoV-2 synthetic RNA and targeting the E1 and N2 genes (Figure 2(H)). Although the maximum signal (Nmax) observed for all tested concentrations, for both CQMED RTs, was found to be equivalent to the reference enzyme (WarmStart RTx) when combined with CQMED *Bst*-LF, it is noteworthy that the amplification start (Cy0) of all reactions containing CQMED *GO*-RT occurred approximately 500 s later compared to the reference reaction and those containing CQMED *HIV*-RT. Considering these results, the combination of CQMED *Bst*-LF at 12 ng/µL with CQMED *HIV*-RT at 2 ng/µL was considered ideal for this RT-LAMP setup.

Enzymes are stable over time, at different storage temperatures, showing high-standard quality parameters: high purity, absence of nuclease contamination, low bacterial DNA carry-over, and consistency in different lots

A series of experiments were conducted to assess the quality of the enzymes produced in-house and to determine their effectiveness, even under adverse storage conditions, based on the guidelines established in EP25-A (Evaluation of Stability of In vitro Diagnostic Reagents)[47] with modifications, and results from previous works.[48–50] The activities of the CQMED enzymes, *Bst*-LF and *HIV*-RT, were evaluated after several weeks of storage in non-ideal temperatures, compared to the enzymes kept at the recommended storage condition of –20 °C. Figure 3(A) demonstrates that average LAMP Ct (Cycle threshold) values ranging from 16 to 20 were observed for CQMED *Bst*-LF across the tested time

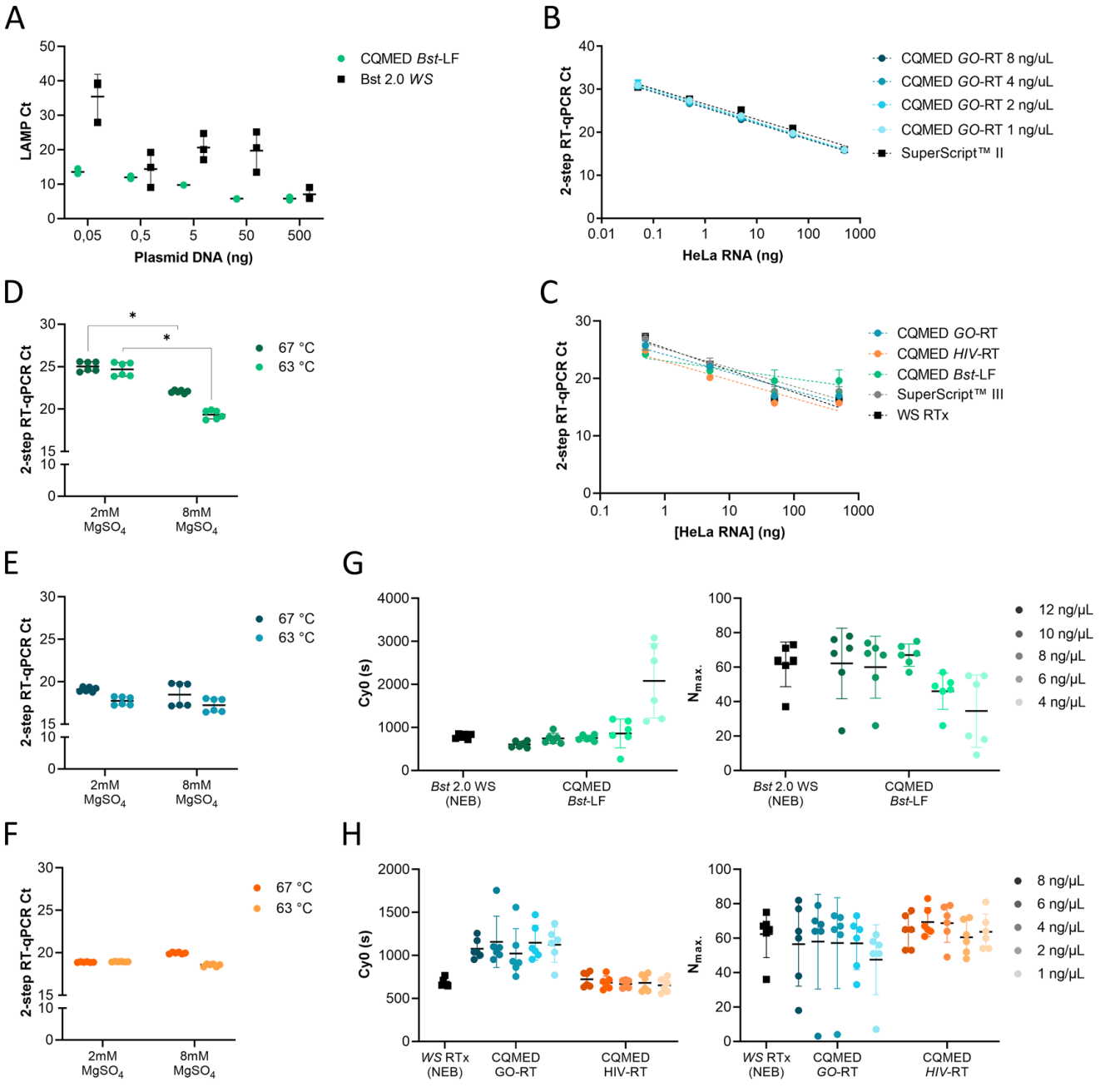

Figure 2. CQMED enzymes are active in comparison to commercial enzymes, and were optimized for RT-LAMP and applied to the point-of-care system. (A) Comparative polymerase activity measurement of CQMED *Bst*-LF (12 ng/uL) with *Bst* 2.0 WarmStart DNA Polymerase (NEB) in LAMP reactions. (B) CQMED *GO*-RT reverse transcription efficiency, at different concentrations and measured by two-step RT-qPCR versus SuperScript II (Thermo). GAPDH cDNA generated by the reverse transcriptase at indicated concentrations from 50 pg–500 ng of HeLa cells total RNA. (C) Comparison of reverse transcription efficiency among the three different CQMED enzymes and the commercial equivalent ones, using two-step RT-qPCR. cDNA synthesis was carried out at 55 °C (63 °C for CQMED *Bst*-LF) for 30 min, with 250 ng of HeLa cells total RNA, isothermal buffer for the CQMED-produced enzymes, and the manufacturer's buffer for the commercial enzymes. Influence of the $MgSO_4$ supplementation and temperature in RT reactions for CQMED *Bst*-LF (D), CQMED *GO*-RT (E), and CQMED *HIV*-RT (F). (G) CQMED *Bst*-LF titration comparison with *Bst* 2.0 WarmStart (NEB) at 0.4 U/µL in LAMP assays: Left graph Cy0 (s); Right graph Nmax (delta). Data were obtained using the Hilab Molecular equipment. (H) CQMED *GO*-RT and *HIV*-RT titration compared to WarmStart RTx Reverse Transcriptase (NEB) in RT-LAMP assay: Left graph Cy0 (s); Right graph N_{max} (delta). Data were obtained using the Hilab molecular equipment. Experiments A and B were performed as one experiment with technical triplicates.

points. Meanwhile, CQMED *HIV*-RT maintained Ct values around 20 in the two-step RT-qPCR assays (Figure 3(B)). In both scenarios, the results remained consistent with the values obtained for the enzymes kept under ideal storage conditions. In addition, CQMED *GO*-RT underwent a similar assessment, exhibiting no significant reduction in activity (Supplemental Figure S3).

In addition to the enzyme stability/resilience tests discussed above, we selected four other parameters as quality indicators of the produced enzymes: protein purity, nuclease activity contamination, residual bacterial gDNA, and DNAPol activity assessment with Unit definition.

The protein purity of the final enzyme stocks was assessed through sodium dodecyl sulfate polyacrylamide

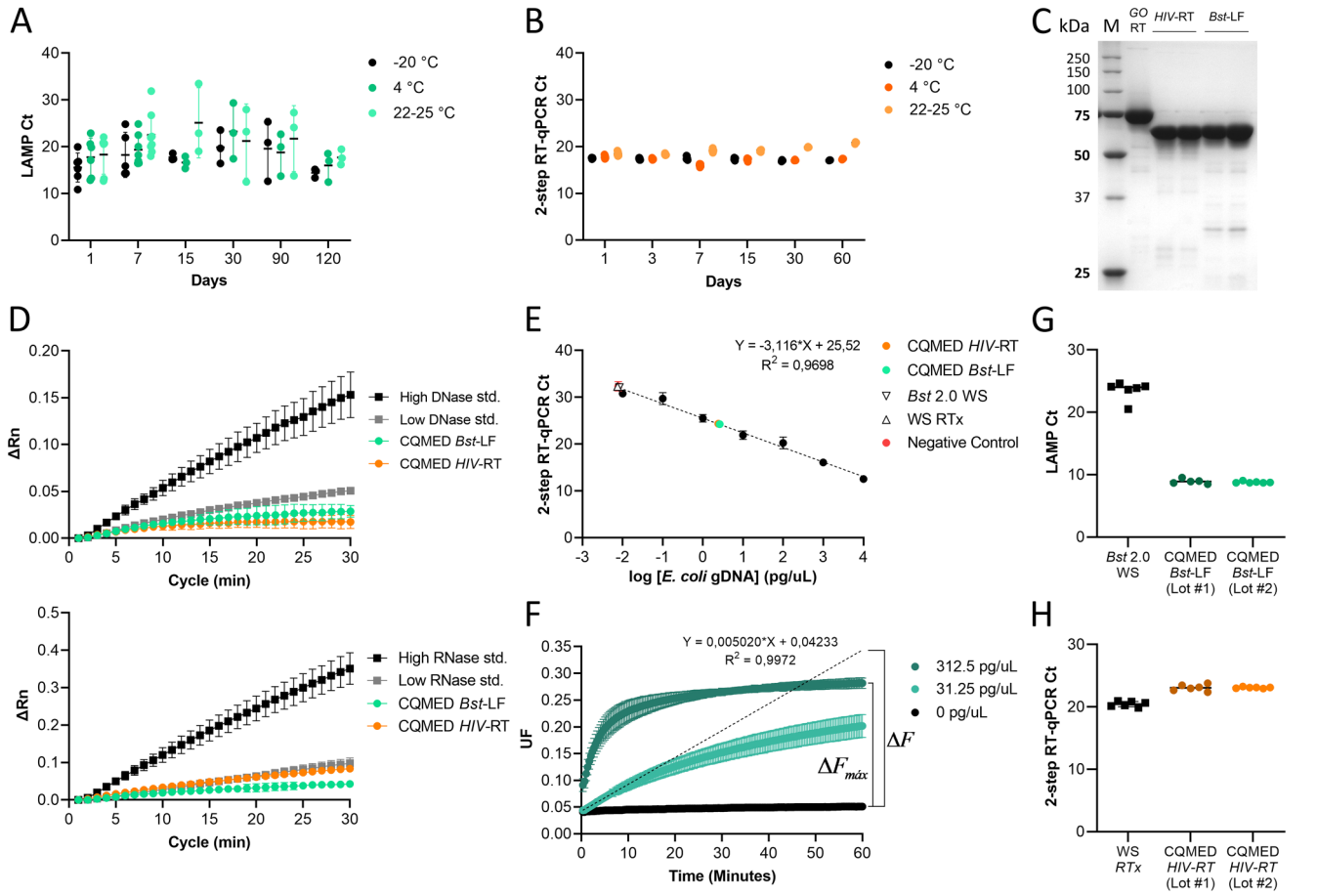

Figure 3. Enzymes stability at adverse storage temperatures and quality control tests. Evaluation of enzymatic stability after storage in adverse temperatures: 4 °C and room temperature (22–25 °C) (A-B): (A) CQMED *Bst*-LF activity measured in fluorescent LAMP reactions containing pUC57 plasmid harboring SARS-CoV-2 genes E1 and N2, at a concentration of 10^6 copies/reaction (500 copies/µL) each; (B) CQMED *HIV*-RT activity measured in RT-qPCR reactions with HeLa cells' total RNA (250 ng) targeting the GAPDH gene. (C) Final products purity and homogeneity assessment by SDS-PAGE gel analysis. 3 µg per well. 12% SDS-PAGE. (D) Kinetic nuclease activity evaluation of CQMED *Bst*-LF and *HIV*-RT ($n = 2$). Enzymes were diluted to a final 2% glycerol concentration, according to the manufacturer's recommendation, and tested in 20 µL reactions at 60 ng/µL for *Bst*-LF and 30 ng/µL for *HIV*-RT. Nuclease activity was qualitatively estimated based on DNase I (upper) and RNase A (lower) activity standards. PCR-grade water was used as negative control (data not shown). Data are representative of just one of the experimental repetitions with reproducible results. High and low DNase standards correspond to 4×10^5 units/µL and 1×10^5 units/µL of DNase I, respectively. High and low RNase standards correspond to 0.4 pg/µL and 0.1 pg/µL of RNase A, respectively. (E) *E. coli* residual gDNA contamination in CQMED *Bst*-LF and *HIV*-RT ($n = 2$) was investigated by qPCR targeting the bacterial 16S gene. The range of contamination in our samples was determined using *E. coli* gDNA serial dilutions as a standard curve. Data are representative of both experimental repetitions with reproducible results. (F) CQMED *Bst*-LF unit determination, Lot#1. Saturating and non-saturating curves for polymerase activity of CQMED *Bst*-LF measured with EvaEZ Fluorometric Polymerase Activity Assay Kit (Biotium) in a QuantStudio 6. 1 U of CQMED *Bst*-LF—the amount of enzyme necessary to incorporate 25 nmol of dNTP in 60 min at 67 °C in a Tris-buffered system containing 2.5 mM Mg^{2+}—corresponds to 44 ng of protein from Lot#1. (G-H) Enzymes resilience test and activity comparison between two production batches generated one year apart from each other, and stored at −20 °C. Activities from A and B were measured in at least one experiment with three technical replicates. The experiment F was executed as two independent experiments with two technical replicates each.

gel electrophoresis (SDS-PAGE) analysis. Figure 3(C) demonstrates that all CQMED enzymes were obtained as single bands, representing highly pure products, and showing reproducibility between different production lots of CQMED *Bst*-LF and *HIV*-RT.

As seen in Figure 3(D) (top) and Supplemental Figure S4(A) and (B), the detected nuclease activity of both CQMED enzymes was below the lower DNase standard. As such, there is little to no residual DNase activity in the preparations, indicating that neither the *Bst*-LF nor *HIV*-RT protein preparations can negatively affect the DNA content generated in the reaction.

Regarding RNase activity, the time-activity curves of CQMED *HIV*-RT exhibited signals in close proximity to the low standard reference of 0.1 pg/µL of RNase A (Figure 3(D) (bottom) and Figure S4(A) and S4(B)). Experimental replicates, including analysis of different protein production lots, are shown in Supplemental Figure S4. CQMED *GO*-RT protein preparation showed no traces of DNase or RNase catalysis (Supplemental Figure S4(C)).

For the *E. coli DNA* contamination assay, three sets of primers targeting the *E. coli* ribosome 16S gene were used. Two of them (sets 1 and 2) were obtained from the literature,[39,40] and the other (set 3) was designed using the Primer-BLAST tool from NCBI. All three sets amplified the target gene accordingly in the PCR reactions (Supplemental Figure S5(A)). In order to eliminate the potential interference of the active polymerases in the amplification reaction, we conducted a PK digestion of all protein samples before conducting the qPCR experiment. The interpolation of the cycle

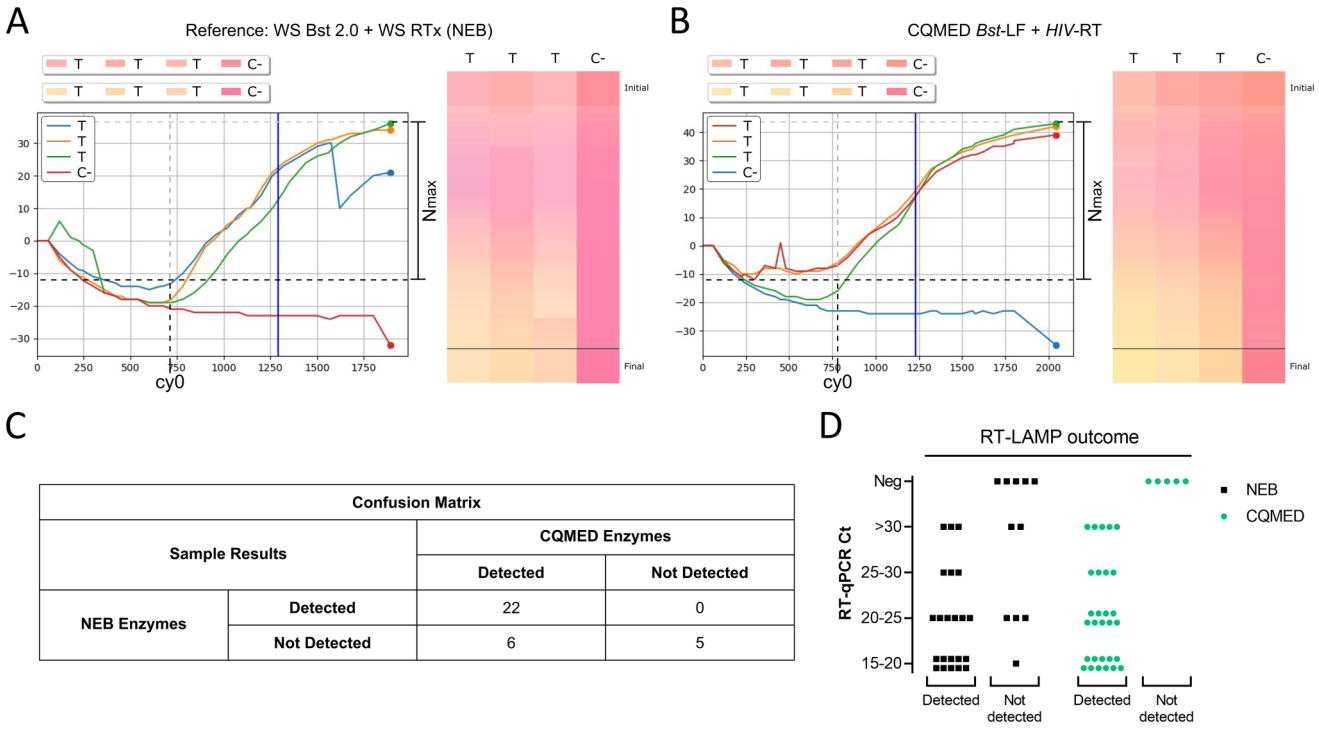

Figure 4. CQMED enzymes applied to colorimetric RT-LAMP in the Hilab Molecular equipment show similar curve patterns and color profiles to those of the reference, and can effectively detect SARS-CoV-2 RNA in clinical samples. The color series and its graphic representation obtained from the Hilab Molecular equipment are displayed above for colorimetric RT-LAMP reactions containing synthetic SARS-CoV-2 RNA and NEB (A) or CQMED (B) enzymes. The confusion matrix (C) and the graphical representation of the assertiveness (D) of Hilab Molecular RT-LAMP reactions with NEB and CQMED enzymes in detecting SARS-CoV-2 RNA in clinical samples are shown.

threshold observed for each enzyme sample to the standard curve containing known amounts of total bacterial DNA allowed the indirect estimation of the residual gDNA in each sample: 2.6 pg/µL for CQMED *Bst*-LF, 2.4 pg/µL for CQMED *HIV*-RT (Figure 3(E)), and 0.01 pg/µL for CQMED *GO*-RT (Supplemental Figure S5(B)). Therefore, the standardized protocol produced enzymes in a carry-over range of up to 3 pg/µL of *E. coli* gDNA.

The activity of CQMED *Bst*-LF was measured using a commercial kit for DNA polymerase activity assessment—EvaEZ Fluorometric Polymerase Activity Assay Kit (Biotium). This kit allows the determination of the enzyme activity, based on the amount of nucleotides incorporated over time, and consequently, the definition of an enzyme unit. A unit system is important to ensure a real correlation between the produced enzyme concentration and activity, enabling accurate measurement, comparison between lots, and understanding of the enzyme activity in different protocols.

We considered 1 U of CQMED *Bst*-LF as the amount of enzyme necessary to incorporate 25 nmol of dNTP in a 60 min reaction at 67 °C, in a Tris-buffered system containing 2.5 mM Mg^{2+}. Accordingly, for the production Lot#1, 1 U corresponds to approximately 44 ng of the enzyme (Figure 3(F)); and for Lot#2, 1 U = 27 ng of CQMED *Bst*-LF (Supplemental Figure S6).

Finally, we compared the activity of two production batches of CQMED *Bst*-LF and *HIV*-RT generated one year apart from each other (Supplemental Figure 3(G) and (H)). The goal was to verify the resilience of the enzymes after long-term storage and the reproducibility of our method. This assay demonstrated no significant differences between the two different lots.

Optimized colorimetric RT-LAMP protocol with CQMED enzymes can effectively detect SARS-CoV-2 RNA in clinical samples

After establishing the protocols to obtain high yields of pure and active enzymes for the RT-LAMP, we tested their use in a colorimetric PoC system (Hilab Molecular device).

Initially, the enzymes were tested in reactions containing synthetic SARS-CoV-2 RNA, comparing their efficiency with the commercially available enzymes (NEB). The color series results for each enzyme and the graphical representation of the RT-LAMP reactions are shown in Figure 4(A) and (B). The curve pattern and color profile observed for both commercial and produced in-house enzyme combinations were very similar, as well as the calculated Cy0 and N_{max} values.

Next, to evaluate the efficacy of the produced enzymes in correctly detecting SARS-CoV-2 in clinical samples, we conducted RT-LAMP reactions on a group of COVID-positive human nasopharyngeal swab samples, previously collected,[29] sorted according to their RT-PCR cycle thresholds (Cts). During this evaluation, we also compared the outcomes from the RT-LAMP reactions using commercial enzymes (NEB) with the outcome of the CQMED enzymes.

The tested group comprised 28 positive samples with Cts ranging from 15 to above 30, along with five negative

patients to test for false positives. The results, presented in a confusion matrix (Figure 4(C)), demonstrate an overall correlation of 82% between the two enzyme sets. Specifically, 22 positive samples were detected by both sets, while the five negative patients were not detected by either. Out of the 33 samples tested, six exhibited discordant results, with one falling within the RT-qPCR Cycle threshold range of 15–20, three between 20 and 25, and two above 30. Notably, the NEB enzyme pair, comprising Bst 2.0 WarmStart DNA Polymerase and WarmStart RTx reverse transcriptase, produced all false-negative results for these discordant cases, as illustrated in Figure 4(D).

Due to the high correlation with the commercial enzymes and the ability to detect the positive samples even with a low viral load (high Ct samples) without generating false positives, we concluded that the enzymes produced in our protocol demonstrate sensitivity and accuracy comparable to commercial enzymes.

Discussion

The COVID-19 pandemic revealed both the fragility of health systems and the capacity of the world's medical community to quickly respond to an emergency. Due to the higher demand for diagnostic tests worldwide, during the pandemic, commercial patent-protected enzymes such as SuperScript II and SuperScript III, widely used for the cDNA synthesis step in RT-qPCR and RT-LAMP reactions, were suddenly unavailable. To mitigate this problem, the scientific community sought to find patent-free alternative enzymes that could be effectively used in place of commercial enzymes and to establish protein purification protocols.[19] Even after the control of the pandemic,[51] scientists continue to optimize processes for local production of reagents and to develop and improve diagnostic techniques, which can be fundamental to fighting new pandemics and to make molecular diagnostics cheaper and broadly available. In this context, our work contributes to these efforts in developing a reliable protocol to achieve high-quality and stable enzymes in good yields for the RT-LAMP reactions and demonstrating their use in a PoC system.

The standardization of the expression and purification of LAMP enzymes indicated that protein expression levels of both enzymes were affected by the tag type and position, leading us to select the N-terminal His-tagged proteins (6xHis-*HIV*-RT and 6xHis-*Bst*-LF) for protein production. The expression from these clones and purification of the corresponding enzymes, using the optimized protocol described in methods, produced a consistently greater yield of enzymes than previously reported,[19] considering the number of tests per liter of the original culture (two folds higher for *Bst*-LF and nine folds higher for the *HIV*-RT). The N-terminal position of the tag appears to be the major factor affecting the yield, although we cannot conclude for certain because we have not tested the original protocol published by Kellner *et al.*

Both enzymes, produced and purified using our protocol, were tested and shown to be pure and highly active. The purified CQMED *Bst*-LF has very similar activity to that previously reported for the standard *Bst* 2.0 WarmStart DNA Polymerase (NEB), in the concentration range of 8–12 ng/μL.[52,53] We did not observe a significant difference between the produced *HIV*-RT and the standard commercial enzymes tested, using a protocol of 10–30 min reaction at 50 °C–55 °C. As the reverse transcriptase activity can vary according to the RT type, template sequence, or reaction conditions,[54,55] it would be important to further optimize the enzyme for difficult templates, especially rich in CG sequences, in case the enzyme is used in other applications. Testing the produced enzymes with real patient samples demonstrates their reliability, comparable to that of commercial enzymes, in detecting COVID-19-positive patients. In our analysis, CQMED enzymes slightly outperformed NEB enzymes, identifying six more COVID-positive patients out of the 28 tested positives, without amplifying the negative controls. Due to the number of samples tested, it is likely that this difference is due to statistical variation. Another possibility is that since the enzymes are not identical, they may exhibit different sensitivities to inhibitors present in the biological samples.[56] However, further tests would be necessary to verify this possibility.

In terms of contaminants with the potential to interfere with either the sensibility or the accuracy of the LAMP reactions, we have tested nucleases and bacterial genomic DNA contamination of the enzymes prepared by our protocol. Of all the produced enzymes, only the CQMED *HIV*-RT prep has ribonuclease activity equivalent to 0.1 pg/μL of RNase A (low standard control). The residual RNase activity observed is probably due to the existence of an RNase H domain in the C-terminal of this protein.[57] As for the bacterial genomic DNA, the amount of gDNA detected is very low (carry-over below 3 pg/μL of *E. coli* gDNA) and does not seem to promote false positives with the tested primers or to reduce the sensibility of the LAMP reaction, based on our comparison with commercial enzymes. If the small amount of gDNA carry-over is a problem for other specific applications, it is possible to include a nucleic acid removal step by DNA precipitation with the positively charged polyethyleneimine (PEI),[58] or by nuclease digestion during the purification process,[59] without drastically changing the enhanced purification protocol pipeline. However, the use of nucleases must be considered carefully in the face of the possible carryover to the final product and consequent influence on reaction outcomes.

Considering a pandemic scenario, and the need for reagents to endure transportation at non-ideal temperatures to places without the infrastructure to keep them properly stocked, enzymes with good resilience and stability are desirable. Therefore, we executed a series of experiments to characterize and investigate whether the selected enzymes are still active after adverse storage conditions in the buffers that we use to stock them. The *Bst-LF* produced by our protocol presented a similar activity, independent of the storage conditions (4 °C, or average room temperature from 22 °C to 25 °C), maintaining a LAMP Ct around 20, even when stored at room temperature up to 120 days. Other studies reported stability of up to 15 days at 25 or 37 °C[49] and up to 45 days at room temperature.[50] For the RTs, both CQMED *HIV*-RT and

CQMED *GO*-RT maintained their activity, with little (room temperature) or no loss of function (4 °C) for up to 60 days of adverse storage, the longest tested condition.

In conclusion, this study offers a reliable and affordable method for locally producing enzymes for molecular diagnostics. The established techniques deliver high-quality proteins with thermal and temporal stability and exhibit activity that is comparable to that of commercial enzymes that meet the highest standards. We offer a practical method for decentralized case monitoring by including these reagents in a PoC system using RT-LAMP. The flexible and user-friendly platform is appropriate for quick deployment even in remote locations and is easily adaptable to various diseases. These qualifiers enable quick epidemic responses in resource-constrained and field situations and are aligned with the optimum diagnostic test standards proposed by the WHO.[60] Furthermore, the enzymes featured in this study—CQMED *GO*-RT, CQMED *Bst*-LF, and CQMED *HIV*-RT—can be applied to traditional systems, in addition to PoC devices. Beyond the constraints of colorimetric LAMP assays, the coupling of LAMP or RT-LAMP with DNA intercalation dyes allows usability in typical qPCR systems. Overall, this work provides a full and adaptable solution to the main issues faced in public health-related molecular diagnostics, enabling the development of efficient and adaptable testing methodologies.

AUTHORS' CONTRIBUTIONS

Conceptualization: LRS, IEPS, MHB, KBM, HPR, MVMF, BMMA; Experimental design, execution and data analyses: LRS, IEPS, GCS, BWR, LMI, EYSK and GVV; Supervision: LRS, MHB, KBM, HPR; Writing—original draft: LRS, IEPS, GCS, BWR, LMI, MHB, KBM; Writing—editing: LRS; Writing—review: All authors participated in the review of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

MVMF is the CEO at Hi Technologies LTDA; SRR is the CTO at Hi Technologies LTDA; BMMA is the CMO at Hi Technologies LTDA. BWR, LMI, EYSK, HPR work or worked at Hi Technologies LTDA and do not declare any competing interest.

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ETHICAL APPROVAL

Ethics Committee Boarding of Complexo Hospital de Clínicas of Universidade Federal do Paraná (CAAE: 31687620.2.0000.0096, approved on 13 December 2020).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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