

Corona Detective: a simple, scalable, and robust SARS-CoV-2 detection method based on reverse transcription loop-mediated isothermal amplification

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Surveillance screening at scale to identify people infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) prior to extensive transmission is key to bringing an end to the coronavirus disease 2019 (COVID-19) pandemic, even though vaccinations have already begun. Here we describe Corona Detective, a sensitive and rapid molecular test to detect the virus, based on loop-mediated isothermal amplification, which could be applied anywhere at low cost. Critically, the method uses freeze-dried reagents, readily shipped without cold-chain dependence. The reaction detects the viral nucleocapsid gene through a sequence-specific quenched-fluorescence readout, which avoids false positives and also allows multiplex detection with an internal control cellular RNA. Corona Detective can be used in 8-tube strips to be read with a simple open-design fluorescence detector. Other methods to use and produce Corona Detective locally in a variety of formats are possible and already openly shared. Detection specificity is ensured through inclusion of positive and negative control reactions to run in parallel with the diagnostic reactions. A simple user protocol, including sample preparation, and a bioinformatics pipeline to ensure that viral variants will still be detectable with SARS-CoV-2 primer sets complete the method. Through rapid production and distribution of Corona Detective reactions, quite inexpensive at scale, daily or weekly surveillance testing of large populations, without waiting for symptoms to develop, is anticipated, in combination with vaccination campaigns, to finally control this pandemic.

KEY WORDS: computational biology, COVID-19, fluorescence, molecular diagnostic techniques, open-source

INTRODUCTION

The recent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has revealed fragility in public health preparedness. The pandemic requires a fair, rapid, large-scale deployment of biologic testing beyond what the existing biomedical infrastructure was able to supply early in 2020.¹ Despite enormous efforts across public and private sectors, detection of SARS-CoV-2 in people or in the environment remains cumbersome and expensive.² Currently used techniques of molecular amplification are costly and require complex infrastructure and biosafety procedures, limiting diagnosis of people everywhere.³ Even with vaccines already being administered, testing remains a crucial factor to control the current pandemic,^{4, 5} with prompt surveillance testing of populations being particularly important, as 40% of potential carriers are asymptomatic⁶ and as new virus variants evolve (nextstrain.org). Sensitive alternatives to quantitative reverse transcription PCR,

the “gold standard” for molecular diagnostics of this RNA virus, with a simpler reaction methodology and reagents that can be readily shipped and stored without cold-chain dependence, are necessary.²

The Corona Detective described herein is based upon reverse transcription loop-mediated isothermal amplification (RT-LAMP). Loop-mediated isothermal amplification (LAMP) rivals PCR for sensitivity, without requiring thermocycling or even a cold chain.^{7, 8} Multiple groups have recently developed RT-LAMP assays for detection of SARS-CoV-2,^{3, 9} (see review in this journal). Because the majority of these reported methods rely on using nonspecific readout methods, such as intercalating dyes or a change of pH, they cannot readily distinguish spurious amplicons that can arise during isothermal amplification,^{10, 11} increasing the risk and rate of false positives.

This shortcoming calls for the use of sequence-specific detection methods,¹² such as the one used in the first RT-LAMP test to receive an Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration¹³ (iAMP COVID-19 Detection Kit) or quenching of unincorporated amplification signal reporters (QUASR).^{14, 15} This method employs a fluorescently tagged primer from

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the main LAMP set [forward inner primer (FIP)/backward inner primer (BIP) or loop forward (LF)/loop backward (LB)] and a short oligonucleotide complementary to the one tagged, but with a quencher that blocks the fluorescence when bound, allowing for fluorescent signals specifically from produced amplicons. This detection strategy improves specificity and allows multiplexing for the detection of more than one target in a single reaction.^{14, 15}

Over recent years, we democratized LAMP-based QUASR detection reactions for use by members of the general public (aged 5–75) to learn about and to detect genetically modified organisms in open science workshops spearheaded in the GMO Detective project.¹⁶ To this end, we optimized speed, sensitivity, and robust use (Aidelberg G, in preparation) and also designed an affordable (less than \$2) and easy-to-build open-source hardware fluorescence detector.¹⁷ This earlier work enabled quick adaptation of the QUASR method for SARS-CoV-2 detection. As an open science collaboration with Just One Giant Lab and its international team of collaborators, including the Center for Research and Interdisciplinarity (Paris, France) and the community lab, Hackuarium (Lausanne, Switzerland), do-it-together participatory research brought together the Corona Detective project.¹⁸ One key innovation of the method, use of freeze-dried reaction components, allows Corona Detective to be shipped anywhere readily, with no cold-chain dependence and in a variety of shelf-stable formats. This means scaling up this method may be truly possible. That the method can be readily adapted to various targets, additionally, means that this work may also provide better pandemic preparedness for the future.

METHODS

RT-LAMP

The polymerases *Bst* LF and *Bst* 2.0 and the RTx reverse transcriptase (the latter two enzymes “WarmStart” versions) were obtained from New England Biolabs (Ipswich, MA, USA) and used with provided buffers as per manufacturer instructions, titrating magnesium and adjusting primer ratios to optimize reactions based upon real-time amplification, using SYBR-Safe (Thermo Fisher Scientific, Waltham, MA, USA) intercalation for detection, by diluting the stock solution (10,000×) 1/250 and using 1.6 μl per real-time reaction, as described by Carter *et al.*⁸ A plasmid encoding the nucleocapsid (N) gene (10006625; Integrated DNA Technologies, Coralville, IA, USA) and a synthetic RNA control for SARS-CoV-2 (NR-52358; BEI Resources, Manassas, VA, USA) were used as positive controls. Reaction volumes were 20 μl total with 4 μl sample input. Total cell RNA was extracted from inner cheek cells with either the Monarch Kit (New England Biolabs) or RNeasy

(Qiagen, Germantown, MD, USA) per manufacturer instructions. Environmental samples (of doorknobs and surfaces) were obtained by wiping with cellulose tissue, and ~10 mm squares were cut and heated to 95°C for 5 min in 100 μl RNase-free water before use in reactions.

QUASR detection

N-target primers^{19, 20} were chosen for further use in QUASR detection. The FIP primer was tagged at its 5' end with fluorescein (FAM), and a short sequence complementary to it, with a quencher (either Black Hole Quencher or Iowa Black FQ Eurofins, Germany and Integrated DNA Technologies, Belgium, respectively) at its 3' end, was also synthesized (sequence 5'CATTGGC CGCA-Q) for QUASR endpoint detection of the amplification product. RNaseP detection was previously described by Curtis *et al.*²¹ and used a HEX fluorescence marker on the LF primer with a complementary 5'CAGCCAT CCACAT-Q quencher. Quencher oligonucleotides were added at 1.5-fold the concentration of their complementary tagged primer. Reactions were cooled to ambient temperature for hybridization (and thus quenching) of unincorporated signal before imaging with blue excitation and an amber filter [with the detector from the GMO Detective¹⁷ or with a *trans*-illuminator or plate reader (fluorimeter) for 96-well plates].

Sample treatments

Validation tests of the QUASR detection reactions were done on inactivated SARS-CoV-2 viral particles (NR-52286; BEI Resources) and also on viral surrogates (Zepto-Metrix, Buffalo, NY, USA), in particular from a “blind” set of samples to test (from the XPRIZE semifinals), with all unknown samples already inactivated.²² The inactivated viral particles were spiked into various matrices (water, PBS, saliva) before subsequent treatments (below). For tests with these samples, the “direct” sample treatment method used 100× TCEP/EDTA solution (0.25 M TCEP, 0.1 M EDTA pH 8), diluted to 1× in the sample for a final concentration of 2.5 mM TCEP/1 mM EDTA, and heated 5 min at 95°C.²³ Four microliters of this solution was added to rehydrated Corona Detective reactions and incubated at 55°C for 10 min of reverse transcription, then 64°C for 45 min of isothermal amplification. Reactions were allowed to cool to ambient temperatures before viewing results. More details can be found in the user protocol²⁴ for Corona Detective.

Bioinformatics workflow

To assess sequence evolution as the pandemic continues, particularly in viral genomic regions covered by Corona

Detective reaction primer targets, the bioinformatics workflow is as follows:

1. Complete and high coverage genomes are collected from the Global Initiative on Sharing All Influenza Data (GISAID) EpiCoV Database (<https://www.gisaid.org/>).²⁵ Only genomic sequences with lengths of 5000 to 32,000 nucleotides were analyzed. The results reported below are from the most recent 2000 submitted sequences for each geographical region (before April 15, 2021).
2. Regional multifasta files are aligned using the MAFFT online service.^{26, 27}
3. The resulting alignment files in CLUSTAL format are analyzed via Python functions. (See the GitHub repository of the project: https://github.com/CoronaDetective/Mutation_Analysis_v1.1.1.)

RESULTS

Reaction design and optimization

Research to develop Corona Detective was openly documented throughout¹⁸ as part of the Just One Giant Lab OpenCovid19 Initiative. Detailed and annotated protocols for making¹⁹ and using²⁴ the Corona Detective are available. In brief, RT-LAMP primer sets targeting the N gene, the most abundant viral RNA in infected cells,²⁸ and also primers targeting cellular human mRNAs for use as internal controls were initially compared. The N gene plasmid and a synthetic RNA control for SARS-CoV-2 were used to optimize reaction conditions, in particular relative primer ratios and magnesium concentrations, while comparing reactions with one of two polymerases, *Bst* LF or *Bst* 2.0.

Two primer sets were chosen for reaction optimization from these early empirical tests: one termed NM, for the viral N gene from the Mammoth protocol,²⁰ and the other, from the RNaseP POP7 gene, termed RP. Examples of real-time data are presented (Fig. 1) with amplification of the control plasmid N gene (Fig. 1A) and reverse transcription and amplification of the control synthetic RNA (Fig. 1B). The *Bst* 2.0 enzyme is more rapid than the original *Bst* LF polymerase, as expected, with faster time to detection threshold (16 vs. 22 min). It also tolerates decreases in magnesium down to 5 mM, unlike *Bst* LF, which would not amplify target under those conditions. Late nontemplate amplification by *Bst* 2.0 was evident, however, although this was only rarely observed with *Bst* LF. Amplification of synthetic target RNA was readily observed, with an apparent limit of detection of about 1 copy per microliter of the Corona Detective reaction or 20 copies (Fig. 1B).

Optimized reaction conditions from these experiments used primer concentrations of 1.6, 0.8, and 0.2 μ M, respectively, for FIP/BIP, LF/LB, and F3/B3, and a magnesium

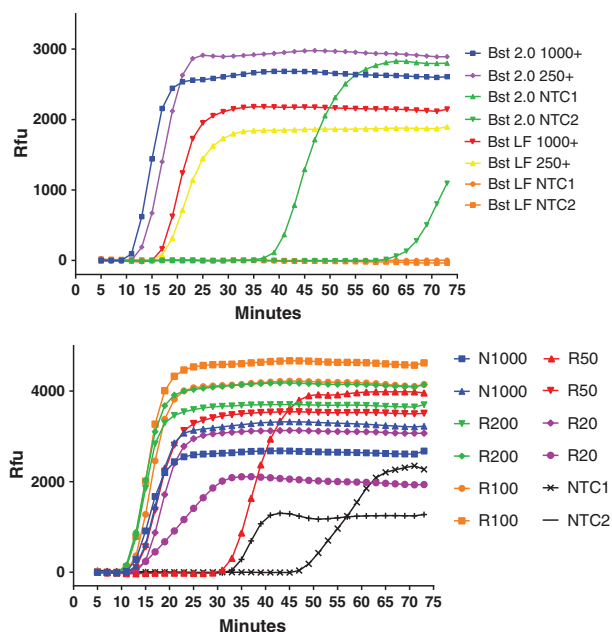


FIGURE 1

Real-time amplification data demonstrate rapid and sensitive detection. A) *Bst* 2.0 is more rapid than *Bst* LF but also more prone to nontemplate amplification. N250/N1000 reactions have 250/1000 copies of the N plasmid control, respectively. Incubation temperature was at 64°C, and reaction volumes were 20 μ l. B) RNA controls are reverse transcribed and amplified for detection down to a single copy per microliter of the reaction. Synthetic RNA (BEI Resources) dilutions were spiked into duplicate reactions at 20, 50, 100, and 200 (R20, R50, R100, and R200) copies, in addition to nontemplate control (NTC) and DNA controls. These reactions used *Bst* 2.0 and RT; incubation included an initial 10 min at 55°C for reverse transcription before shifting up to 64°C for the real-time acquisition. Rfu - relative fluorescence units.

concentration of 7 mM, while aiming to avoid nontemplate control amplification (Table 1).

Sequence-specific fluorescence detection

We designed QUASR primers for the NM primer set (NM*FAM), adding the FAM fluorescent tag to the 5' end of the FIP primer. A short complementary quencher primer, of 11 bases, with the quencher at its 3' end was also synthesized. When annealed to the tagged FIP primer, this quencher prevents fluorescence, but when the FIP is incorporated in the amplicon, this tag fluoresces freely, providing the basis for strong QUASR detection. The internal human control, RP, was detected via a QUASR design used by others already,²¹ with a 13mer attached to a quencher, complementary to the 5' end of the RP LF primer, tagged with HEX, for the RP primer set RP*HEX, used in the multiplex reaction with NM*FAM.

The QUASR fluorescence cannot be read at amplification temperatures but requires lower temperatures (less

TABLE 1

Magnesium optimization of Corona Detective. Concentrations of MgSO₄ were varied in reactions with each of the two polymerases (*Bst* LF or *Bst* 2.0). The fastest time to threshold in minutes for 1000 copies of the N plasmid (NI1000) in a 20 μl reaction is reported.

MgSO ₄ (mM)	<i>Bst</i> LF (min)	<i>Bst</i> 2.0 (min)	Notes
5	na	27	
6	35	21	
7	31	19	Both NTCs up for <i>Bst</i> 2.0 but not <i>Bst</i> LF
8	21	16	Both NTCs up for <i>Bst</i> LF and <i>Bst</i> 2.0

NA, no amplification; NTC, nontemplate control.

than ~35°C) to allow the quencher to anneal to unincorporated tagged primers and prevent fluorescence. It is thus used for endpoint detection. With the simple fluorescence detector from the GMO Detective project,^{16, 17} illuminated by blue LEDs and with amber filters in front of the tubes, controls were readily detected after 10 min incubation at 55°C and 30–90 min at 64°C (Fig. 2). Furthermore, detection of both targets in the reactions was sensitive and reliable, with a limit of detection of 20 RNA copies per reaction for the reaction with NM*FAM (Fig. 2A). In this detector, the HEX signal is somewhat more orange than the green FAM, particularly by eye, and the multiplex reactions, including the NM*FAM and RP*HEX primer sets, are indeed visible in the simple detector (Fig. 2 B, D). A standard phone camera is sufficient to capture images, another advantage of the QUASR method (Fig. 2).

The most encouraging finding from these multiplex QUASR results was that the signals came up well with high sensitivity, but without problems of false positives, even though the *Bst* 2.0 enzyme was used for these reactions, rather than the slower *Bst* LF, and even with incubation of up to 2 h. Overall, complete elimination of false positives (to date, in over 200 negative control reactions) provides great confidence in this QUASR detection. Furthermore, results are stable for at least 1 month when stored in a cool dark place (Fig. 2C). An additional strength of this detection method includes its affordability, with cost per reaction of about \$1 or even less at scale. As mentioned, this can be done using an open-source²⁹ do-it-yourself fluorescence detector for 8-strip tubes¹⁷ (Fig. 2A, B and D), which costs only \$1–\$2 to make. For whole 96-well plates, off-the-shelf blue light transilluminators affordably and easily detect the fluorescent signal (e.g., <https://iorodeo.com/> or <https://www.minipcr.com/>) (Fig. 2C), or if available, a standard gel imaging system or qPCR machines can also be used, all at room temperature.

Sample preparation and reaction specificity and sensitivity

For rapid, affordable, and simple screening especially in lower resource settings, we decided to focus on so-called

“direct” saliva sample processing methods.⁵ The TCEP method²³ for rapidly inactivating and opening virions and reducing viscosity of saliva samples worked best. Benefits of the TCEP strategy include the low volume needed, which prevents sample dilution (1:100), use of only one additional temperature (5 min at 95°C), and the buffer’s room temperature stability. A single copy of the synthetic RNA (BEI Resources) per microliter of reaction was readily detected for these minimally processed samples in Corona Detective assays. The limit of detection for this assay was also determined with viral surrogate particles (ZeptoMetrix) in various matrices (saliva, PBS) and with actual inactivated viral particles (BEI Resources) added prior to TCEP treatment to either PBS or saliva. Limits of detection of 2 copies of SARS-CoV-2 viral particles per microliter of reaction were consistently observed, *i.e.*, 10 copies/μl in the original sample (Fig 2D). For a panel of 30 other viral RNA controls [Twist synthetic RNAs (Twist, South San Francisco, CA, USA), *e.g.*, measles, mumps, influenza, and other coronaviruses], no cross-reactivity was observed, in concordance with the EUA reports^{30, 31} for the NM primer set.

Improved sensitivity may be achieved by using larger input volumes of treated sample per reaction or preconcentrated viral RNA from samples, using commercial kits³² or magnetic beads.^{33, 34} An affordable glass milk method²³ did not scale well to the 96-well microplate format in our hands. To note: sample collection has its own set of issues, and additional biosafety concerns exist until the samples are at least inactivated.

Freeze drying and manufacture

To reduce costs associated with transport, storage, and manipulations and to allow use even where no cold chain is available, reaction components were lyophilized. Briefly, glycerol-free enzymes, along with dNTPs, primer mixes, and trehalose as protein stabilizer,³⁵ were freeze-dried.^{8, 19} The rehydration buffer containing isothermal amplification buffer, MgSO₄, and water is also stable at room temperature (for more detail see Aidelberg and Aronoff¹⁹), and provided together with the reaction tubes. These reaction tubes were found to retain sensitivity and specificity for at least 2

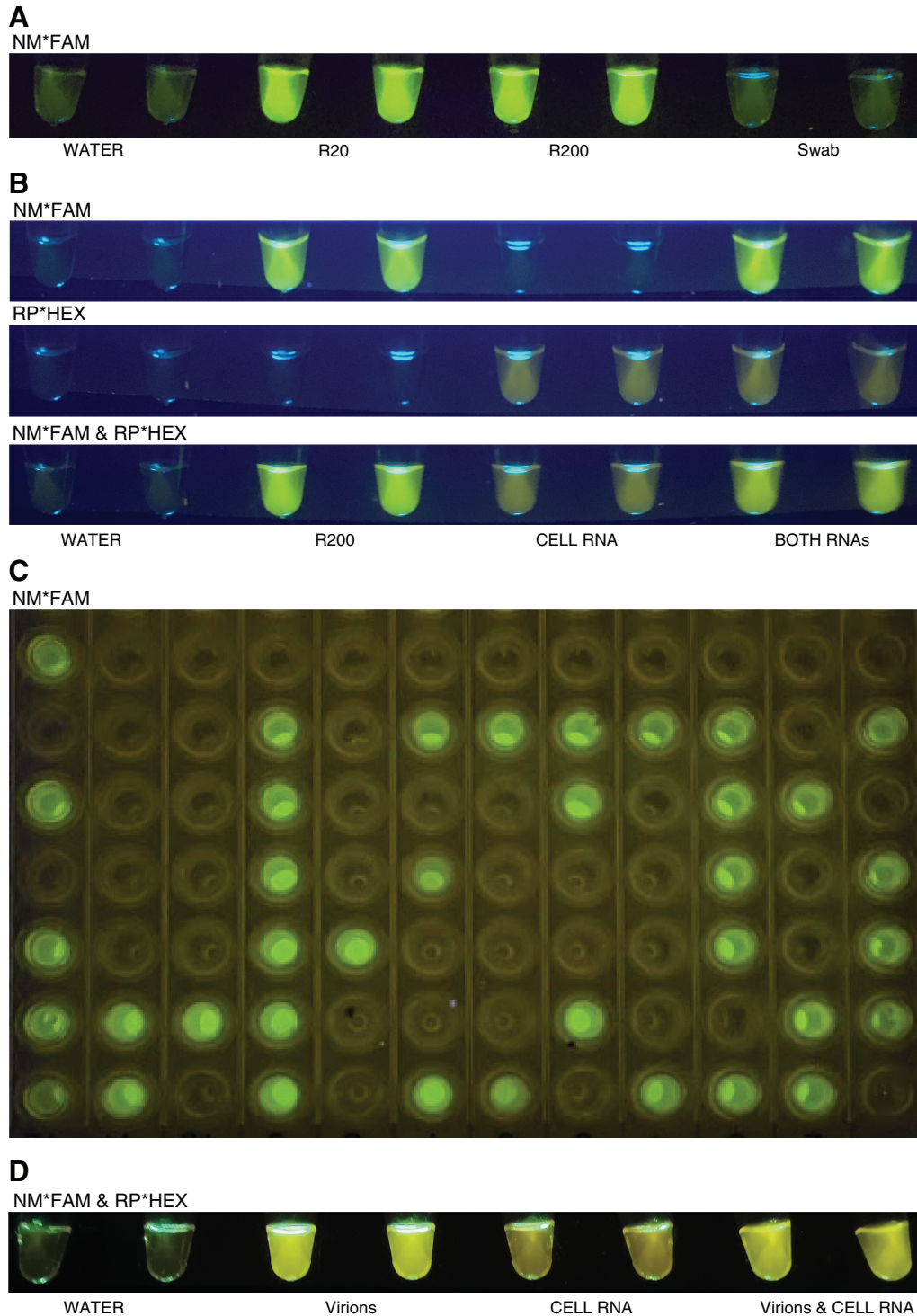


FIGURE 2

QUASR detection for Corona Detective reactions is affordable and unambiguous. *A*) Corona Detective gives positive results down to 20 copies of the synthetic RNA (R20; BEI Resources) per reaction, but no positive reactions have yet been observed from environmental swabs. NM*FAM (the NM primer set including the FIP primer tagged with FAM and the complementary quencher). *B*) The multiplex reactions (NM*FAM and RP*HEX, bottom) sum the single reactions, above, and are distinguishable even in this simple ~\$1 do-it-yourself open fluorescence detector. Above-NM primers labeled with FAM; middle-RP primers labeled with HEX; bottom-multiplex reactions with both primer sets. *C*) A 96-well plate imaged on an affordable blue light gel box transilluminator, 1 mo after reactions were run. *D*) Freeze-dried reaction tubes for the multiplex run with minimally treated inactivated virus input (NR-52286; BEI Resources), human cell RNA, and both together. Images acquired with standard smartphones. Reactions run in duplicate with water (nontemplate control), R20 and R200 (20 or 200 copies of synthetic RNA), swab (environmental surface samples), cell RNA (extracted human cheek cell RNA), both RNAs (R200+Cell RNA), and virions (40 copies of heat inactivated virus).

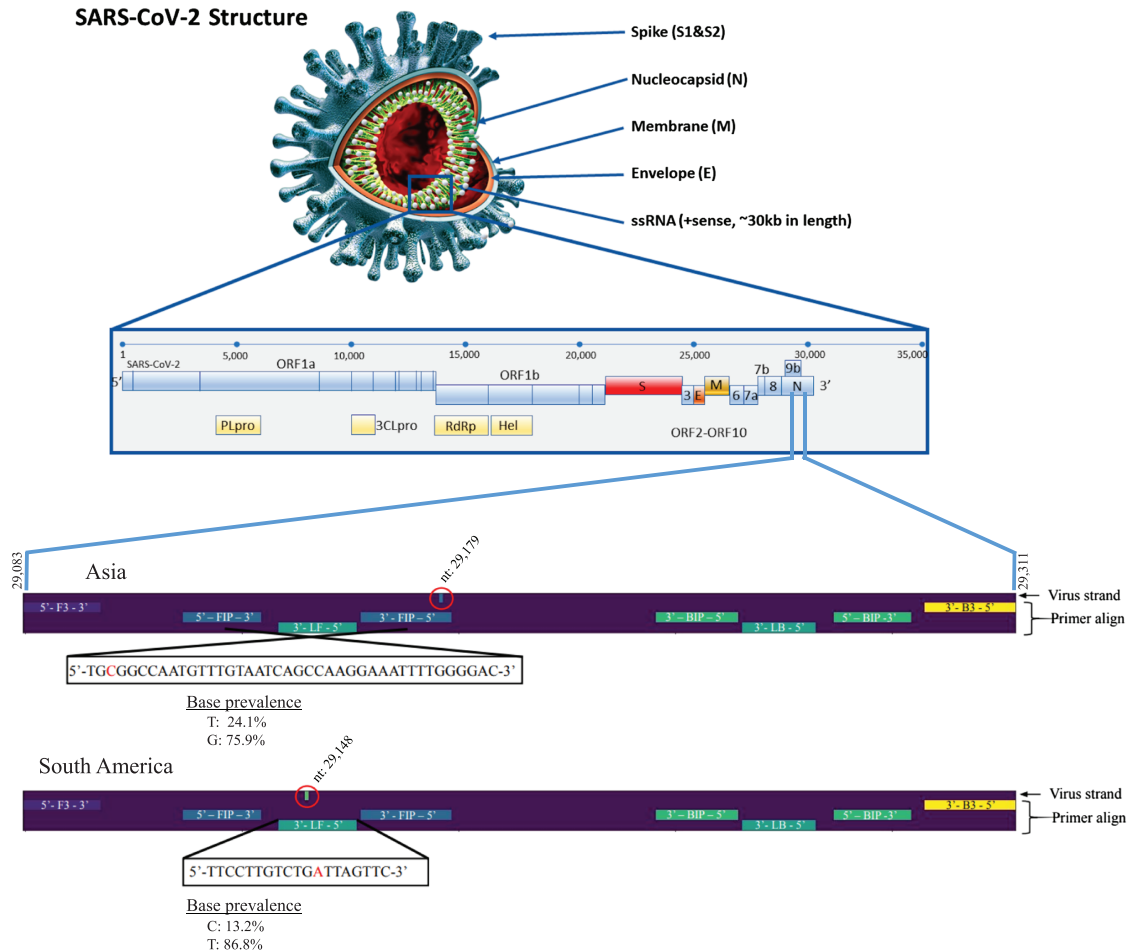


FIGURE 3

Geographic mutational analysis of sequence variation reveals rare changes in two NM primer targets. The SARS-CoV-2 virion structure and genomic organization is shown above (adapted from Kubina and Dziedzic⁴⁴ under Creative Commons Attribution 4.0 International). Bioinformatic analyses from the collected GISAID sequences identified mutations in the N gene of Asian and South American sequenced variants, which are included in two different NM primers, FIP and LF. Site numbering and genome structure use Wuhan-Hu-1/2019 (GenBank: MN908947.3) as the reference sequence. Red circles highlight the position of the nucleotide changes in the viral strand. Base prevalence of mutant and wild-type viral sequences are reported as percentages of the number of sequences analyzed (1908 for Asia, 1930 for South America).

months when sealed with a desiccant and stored in a cool dark location (Fig 2D). Freeze-dried batches have already been utilized after shipping to Sri Lanka at ambient temperatures, with control and contrived sample reactions giving expected results, even after over a month on the shelf.

Bioinformatics

In order to make sure prevalent strains of SARS-CoV-2 will still be detectable by the NM primer set, a bioinformatics pipeline has been developed by the team. A 3-step protocol checks for mutations that might affect recognition by diagnostic primer sets. Results of these analyses, comparing geographical regions, to determine whether any prevalent viral variant may present problems for Corona Detective reactions are shown in Fig. 3.

The NM primer set target sequences are conserved in over 90% of sequenced isolates from GISAID,²⁵ with the exceptions of the Asian and South American SARS-CoV-2 subsets, where a single nucleotide was mutated in two different NM primer recognition sites. For the most recent analyses (database from April 15, 2021), in the Asian sequences a G is mutated to T in the FIP primer target in 24% of sequenced genomes, whereas for the South American sequences a T is mutated to C in the LF primer target in 13% of sequenced genomes (Fig. 3). The rest of the geographic regions analyzed did not present any relevant mutations (over 10% prevalence) for the Corona Detective primers in local variant strains.

To note: potential bias in sequencing clusters, globally, means these analyses are certainly not a definitive measure of all the virus evolving currently.

Since May 12, the last run in GitHub, many more variants are detected, additionally, and NEB has a new on-line tool (<https://primer-monitor.neb.com/>) for similar analyses, in which the NM primer set is called ‘Broughton N’ with BIP/FIP swapped (as in the reference).

DISCUSSION

Done without complex equipment and with a simple fluorescence readout, Corona Detective is an easy-to-use, sensitive, robust, and highly specific SARS-CoV-2 detection method. The RT-LAMP primers chosen for the multiplex detection (including the cellular mRNA target as an extraction control) have already been approved for use in two EUAs from the U.S. Food and Drug Administration^{30, 31} based on RT-LAMP, but with different detection strategies (CRISPR-based and pH-colorimetric, respectively). Drawbacks of these methods, for instance, risks of cross-contamination and sensitivity to buffer effects, are avoided with the sequence-specific quenched-fluorescence-based detection of the QUASR method. Even if the reaction is left to incubate longer than 45 min, false positives in non-template controls are avoided. This is crucial for testing in resource-strapped or point-of-care settings.

Controlling for primer reliability through the described pipeline is so far only done *in silico* but should help ensure robust detection worldwide. Potential problems in amplification for new variants means that vigilance to follow their evolution with bioinformatic algorithms, as we describe here, is highly recommended. Primer sets may require redesign, eventually, to ones that target more preserved genomic regions. Alternatively, two methods can be considered: 1) degenerate or “wobble base” nucleotides, if substituted into problematic positions of useful primer sequences, could allow them to still interact with the target; 2) the addition of a small amount of a high-fidelity polymerase may allow the Corona Detective to be less sensitive to mismatches, as has been described.³⁶ Additional methods could be used, if, on the contrary, being able to distinguish different strains is desirable.^{37, 38}

Use of the freeze-dried reactions after shipment to Sri Lanka under ambient conditions was successful, and further tests for reaction reliability are in progress. More batches of tubes and 96-well plates will be sent soon to Switzerland, Cameroon, Ghana, Chile, Sri Lanka, and potentially more collaborators, made at the Center for Research and Interdisciplinarity in Paris for clinical validation in a variety of contexts. Possibilities for production at scale are already being explored, also in the United States. To further improve Corona Detective, work has begun that aims to utilize in-house-produced, enhanced,³⁹ and/or off patent enzymes (both polymerases and reverse transcriptases^{34,}

^{40, 41}). Additionally, *in silico* optimization studies are in progress by our open science team to increase heat stability and salt tolerance of the polymerase. A point mutation in the tight-binding pocket, anticipated to be more tolerant to modified nucleotides and particularly important for cross-contamination prevention through dUTP incorporation and UTP-glycosidase treatment, will also be examined *in vitro*.

Bottlenecks to rapid scale-up of testing include administrative delays relating to intellectual property and the conservative production infrastructure designed to efficiently supply market needs with limited capacity. These limitations could in principle be overcome through a testing supply chain based upon open-source principles in a distributed, decentralized, and local way.⁴² The tools for high-volume Corona Detective production¹⁹ cost less than an average quantitative PCR machine and could even incorporate locally produced enzymes^{34, 40, 41} or nucleotides.⁴³ Adopting the open Corona Detective in its various formats (8-well, 96-well, 384-well) could help scale up SARS-CoV-2 surveillance, particularly in low-resource settings, sooner rather than later.

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