

PRIMERS

The present application claims priority to Singapore patent application number 10202009456Y titled "Primers)" which is incorporated by reference herein in its entirety.

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Technical Field

The invention relates to primers. In particular, the invention relates to primers that can be used for the detection of SARS-CoV-2 RNA.

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Background

The COVID-19 pandemic is caused by the highly infectious SARS-CoV-2 virus. Mass-testing with easy-to-use diagnostics and surveillance technologies is necessary for controlling the spread of this infectious disease. These technologies that detect the SARS-CoV-2 RNA genome and transcripts are urgently needed across the world. Sensitive and
15 specific detection of SARS-CoV-2 directly impacts clinical and public health outcomes amidst the COVID-19 pandemic.

A highly attractive technology is loop-mediated isothermal amplification (LAMP), which uses sets of 4, 6, or more oligonucleotide primers to amplify desired regions of a target
20 nucleic acid sequence (Notomi et al., Nucleic Acids Research, 2000). LAMP amplifies DNA, whereas coupling of LAMP to reverse transcription gives RT-LAMP that amplifies RNA; this disclosure uses the terms "LAMP" and "RT-LAMP" interchangeably. This isothermal reaction is carried out at a constant temperature in a single tube, which distinguishes LAMP from polymerase chain reaction (PCR) that typically requires cycling
25 a reaction through a series of steps at different temperatures. Importantly, this means that LAMP can be carried out without the need for equipment like thermal cyclers (as required for PCR), which makes LAMP a highly attractive and cost-effective solution for amplification and detection of nucleic acid material from pathogens in a point-of-care/point-of-collection testing (POCT) setting. However, due to strict design constraints on LAMP
30 primers, they cannot be as easily designed unlike that for PCR primers; instead, LAMP primer design typically requires specialized design tools and often do not produce productive primer sets.

LAMP uses four primers mainly the forward internal primer (FIP), the forward outer primer (F3), the backward inner primer (BIP), the backward outer primer (B3). One or both loop primers (LF and LB) may be included to speed up the nucleic acid amplification by providing an increased number of starting points for the replication process to commence at one or both ends of the strand being replicated. In the art, the “forward” term refers to the 5’ end while the “backward” term refers to the 3’ end of the DNA or RNA strand.

Summary

In a first aspect of the invention, there is provided a primer set for a nucleic acid amplification analysis to detect a SARS-CoV-2 virus, the primer set is selected from the group consisting of:

- (a) Set 12: SEQ ID No. 1, SEQ ID NO. 2, SEQ ID No. 7 attached to SEQ ID No. 8 by a first linker, and SEQ ID No. 9 attached to SEQ ID No. 10 by a second linker, wherein the first linker and second linker each independently has 1 to 100 nucleotides, wherein the first linker does not consist of a first sequence complementary to a first nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 7 and SEQ ID No. 8, wherein the second linker does not consist of a second sequence complementary to a second nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 9 and SEQ ID No. 10;
- (b) Set 10: SEQ ID No. 11, SEQ ID NO. 12, SEQ ID No. 17 attached to SEQ ID No. 18 by a third linker, and SEQ ID No. 19 attached to SEQ ID No. 20 by a fourth linker, wherein the third linker and fourth linker each independently has 1 to 100 nucleotides, wherein the third linker does not consist of a third sequence complementary to a third nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 17 and SEQ ID No. 18, wherein the fourth linker does not consist of a fourth sequence complementary to a fourth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 19 and SEQ ID No. 20;
- (c) Set 20: SEQ ID No. 21, SEQ ID NO. 22, SEQ ID No. 27 attached to SEQ ID No. 28 by a fifth linker, and SEQ ID No. 29 attached to SEQ ID No. 30 by a sixth linker, wherein the fifth linker and sixth linker each independently has 1 to 100 nucleotides, wherein the fifth linker does not consist of a fifth sequence complementary to a fifth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 27 and SEQ ID No. 28, wherein the sixth linker does not consist of a sixth sequence complementary

to a sixth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 29 and SEQ ID No. 30; and

(d) any combinations of each set above. Thus, primer sets 12, 10 and 20 may be used alone or combined in any combinations thereof.

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Thus, each linker in the FIP and BIP primer may contain 1 to 100 nucleotides but not the reverse complement of the natural sequence in the SARS-CoV-2 virus between the two regions that binds to the two binding regions of the respective FIP and BIP primer in each primer set.

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Each primer set selected preferably consists essentially of or consists of the sequences in the SEQ IDs. Preferably, Set 12 further comprises SEQ ID No. 3 and/or SEQ ID No. 4; Set 10 further comprises SEQ ID No. 13 and/or SEQ ID No. 14; and Set 20 further comprises SEQ ID No. 23 and/or SEQ ID No. 24. Advantageously, the inclusion of the loop primers increases the amplification speed. More preferably, the primer set is selected from the group consisting of: Set 12: SEQ ID Nos. 1 to 6; Set 10: 11 to 16; and Set 20: 21 to 26; or a combination of any two or more of these primer sets. Advantageously, the primer sets alone are as effective as existing primers while the combination of the primer sets provide a significant improvement over the existing primers for the positive detection of the targeted viral sequence at low concentrations. This may lead to early detection of people infected with the virus possibly before symptoms are observed and allows for the infected person to be isolated more quickly to prevent the spread of the virus.

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Further provided is a composition for detecting a SARS-CoV-2 virus, comprising a primer set according to the first aspect; a DNA polymerase; and a reverse transcriptase. Further provided is a kit for detecting a SARS-CoV-2 virus, comprising a primer set according to the first aspect; a DNA polymerase; a reverse transcriptase; and a positive control.

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In a second aspect of the invention, there is provided a method to detect a SARS-CoV-2 virus in a sample, the method comprising: (a) providing a primer set according to the first aspect of the invention; (b) carrying out a nucleic acid amplification reaction using the sample and the primer set to produce a reaction product mixture; and (c) analysing the reaction product mixture to determine the presence of the SARS-CoV-2 virus in the sample.

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The method may be practiced with existing laboratory equipment with the primer set, composition or kit described above.

Detailed Description

5 In the figures:

Figure (Fig.) 1 shows a schematic of the new primers designed against the SARS-CoV-2 RNA.

Figure 2 shows the colorimetric RT-LAMP reaction replicates with published primers (prior art) at 100 copies/reaction of Twist synthetic RNA controls vs. NTC (0 copies/reaction).

10 Results here were collated from multiple replicates across different days for an initial screen of the best performing prior art.

Figure 3 shows the colorimetric RT-LAMP reaction replicates with 20 sets of computationally designed primers at 0, 10, 50 and 100 copies/reaction of Twist synthetic RNA controls.

15 Figure 4 shows the RT-LAMP reactions with NO series LAMP primer sets against varying concentrations of Twist synthetic RNA controls. Reactions that showed clear positive colour changes from pink to yellow are represented with dark grey squares; reactions that showed partial colour changes from pink to orange are represented with light grey squares; reactions that showed no discernible colour changes and remained pink are represented with white squares.

20 Figure 5 shows the Limit of Detection (LoD) determination of the best-performing primer sets – existing LAMP primer sets As1e, NEB E1 and N2, and the LAMP primer sets 10, 12 and 20. All 6 primer sets were tested at 50, 75 and 100 copies/reaction of synthetic RNA controls at 40 replicates per primer set per concentration of controls. For each concentration of synthetic RNA controls assayed, all 6 primer sets were tested on the same day in replicates of 20, using the exact same physical set of diluted synthetic RNA controls for a more reliable comparison across primer sets. Each primer set was assayed at the NTC condition for a total of 20 replicates as well across multiple days.

25 Figure 6 shows the comparative characterisation of multiplexed LAMP primer sets for existing primers and the new primers.

In the following description, numerous specific details are set forth in order to provide a thorough understanding of various illustrative embodiments of the invention. It will be understood, however, to one skilled in the art, that embodiments of the invention may be

practiced without some or all of these specific details. Embodiments described in the context of one of the methods or devices are analogously valid for the other methods or devices. Similarly, embodiments described in the context of a method are analogously valid for a device, and vice versa.

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This invention may be practiced within the skill of those skilled in the art using conventional techniques for cell biology, cell culture, molecular biology, gene transformation techniques, microbiology, DNA recombinant techniques, unless otherwise indicated. In addition, a more detailed description of common technique may be found in the following references:

10 Molecular Biotechnology (Bernard et al., ASM press 2014); Molecular Cloning, A Laboratory Manual, 4th Ed. (Sambrook et al., Cold Spring Harbor Laboratory Press 2012); Short Protocols in Molecular Biology, 5th Ed. (Ausubel F. et al. eds., John Wiley & Sons 2002).

- 15 Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs.

The terms “about”, “approximately”, “substantially” must be read with reference to the context of the application as a whole, and have regard to the meaning a particular technical

20 term qualified by such a word usually has in the field concerned. For example, it may be understood that a certain parameter, function, effect, or result can be performed or obtained within a certain tolerance, and the skilled person in the relevant technical field knows how to obtain the tolerance of such term.

- 25 The phrase “at least one of A and B” means it requires only A alone, B alone, or A and B, i.e. only one of A or B is required.

It will be further understood that the endpoints of each of the ranges described herein are significant both in relation to the other endpoint, and independently of the other endpoint. It

30 is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

Compositions in matter of LAMP primer sets that specifically and efficiently amplify SARS-CoV-2 genomic targets and the uses thereof are described herein, particularly in the embodiment of COVID-19 diagnostics and surveillance in the form of a SARS-CoV-2 RNA molecular assay.

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The design principle starts from *in silico* bioinformatics. Three freely available LAMP primer design tools have been described in published literature. These are: Primer Explorer v5 (Eiken Chemical Co., Ltd; <https://primerexplorer.jp/lampv5e/>), LAVA (Torres et al., BMC Bioinformatics, 2011) and GLAPD (Jia et al., Frontiers in Microbiology, 2019).

10 However, these LAMP primer design tools are limited and often non-user-friendly when implemented as standalone tools. Using a new LAMP primer design pipeline, candidate LAMP primer sets that consider multiple design principles and parameters were generated. Our LAMP primer design pipeline used a script wrapper to produce LAMP primer sets from LAVA and GLAPD with recommended LAMP primer thermodynamics parameters. These
15 candidate LAMP primer sets were then filtered as part of the new pipeline for primer sets that fulfill key diagnostics needs, including the inclusivity of SARS-CoV-2 clades via conserved SARS-CoV-2 genomic sequences (assessed via a sequence conservation score) and specificity to SARS-CoV-2 against other related coronaviruses and distant viruses (assessed via an off-target score). The 20 primer sets were designed to be as specific as
20 possible based on sequence differences/similarities of off-targets to on-targets. The design also takes into consideration virus sequence evolution and/or conservation. The combination of which is typically not considered in primer design. Through this, a list of 20 candidate LAMP primer sets was shortlisted for further characterization. The sequences of the 20 primer sets are provided in Table 2 at the end. The primers may be synthesized by any known
25 method and is not elaborated further.

To compare the bioinformatics-guided design process with a routine manual process, several sets of LAMP primers were designed manually ‘by eye’ through an approach that still considered well-established primer design parameters e.g. annealing temperature of the
30 primers, GC content, and guidelines suggested by Eiken Primer Explorer v5 regarding distances between the different primer binding sites on the template for each LAMP primer set. These manually designed primer sets are labelled as the ‘NO’-series.

In various embodiments, the invention relates to compositions of matter for LAMP primers that are used for detecting the presence or absence of RNA from SARS-CoV-2, the virus that causes COVID-19. The technology describes the sequences of primer oligonucleotides that bind to and effect isothermal amplification from the SARS-CoV-2 RNA, particularly with Loop-mediated isothermal AMPLification (LAMP). Each primer set consists of 6 primer sequences. The examples demonstrate that the design of LAMP primers are not obvious, since 27 out of 31 (~87%) tested primer sets fail to produce robust detection performance (detection threshold of approximately ≤ 100 copies of target RNA per 25 μ l reaction volume). This technology discloses 3 primer sets that detect SARS-CoV-2 robustly, with a multiplex primer combination (LoD ≤ 50 copies per reaction) exhibiting superior performance to the best-performing prior art. This composition of matter can be used in diagnostics, surveillance, and detection kits for SARS-CoV-2 RNA.

In a first aspect of the invention, there is provided a primer set for a nucleic acid amplification analysis to detect a SARS-CoV-2 virus, the primer set is selected from the

(a) Set 12: SEQ ID No. 1, SEQ ID NO. 2, SEQ ID No. 7 attached to SEQ ID No. 8 by a first linker, and SEQ ID No. 9 attached to SEQ ID No. 10 by a second linker, wherein the first linker and second linker each independently has 1 to 100 nucleotides, wherein the first linker does not consist of a first sequence complementary to a first nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 7 and SEQ ID No. 8, wherein the second linker does not consist of a second sequence complementary to a second nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 9 and SEQ ID No. 10;

(b) Set 10: SEQ ID No. 11, SEQ ID NO. 12, SEQ ID No. 17 attached to SEQ ID No. 18 by a third linker, and SEQ ID No. 19 attached to SEQ ID No. 20 by a fourth linker, wherein the third linker and fourth linker each independently has 1 to 100 nucleotides, wherein the third linker does not consist of a third sequence complementary to a third nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 17 and SEQ ID No. 18, wherein the fourth linker does not consist of a fourth sequence complementary to a fourth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 19 and SEQ ID No. 20;

(c) Set 20: SEQ ID No. 21, SEQ ID NO. 22, SEQ ID No. 27 attached to SEQ ID No. 28 by a fifth linker, and SEQ ID No. 29 attached to SEQ ID No. 30 by a sixth linker, wherein the fifth linker and sixth linker each independently has 1 to 100 nucleotides, wherein the

fifth linker does not consist of a fifth sequence complementary to a fifth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 27 and SEQ ID No. 28, wherein the sixth linker does not consist of a sixth sequence complementary to a sixth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 29 and SEQ ID No. 30; and

(d) any combinations of each set above. Thus the primer sets 12, 10 and 20 may be used individually or in any combinations thereof. The linkers of each FIP and BIP primer is not complementary to the nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to the binding regions of the respective FIP and BIP primer. In other words, the linker is not a nucleotide sequence that is the reverse complement of the sequence between the two binding regions in the SARS-CoV-2 virus.

By “linker” is meant any useful multivalent (e.g., bivalent) component useful for joining to different portions or segments. Exemplary linkers include a nucleic acid sequence, a chemical linker, etc. The linker may be varied with different nucleotide sequences while still retaining the efficacy of the FIP and BIP primers as long as the two binding regions of the FIP and BIP primers are intact. For example, barcoded FIP primers have been used in LAMP (see Ludwig, K.U., Schmithausen, R.M., Li, D. et al. LAMP-Seq enables sensitive, multiplexed COVID-19 diagnostics using molecular barcoding. *Nat Biotechnol.* (2021). <https://doi.org/10.1038/s41587-021-00966-9>). For example, the linker may have a length of 1 to 100 nucleotides. In some embodiments, the linker has 4 nucleotides. Other exemplary linkers include polyethylene glycol, an alkane chain, an alkyne group, a click-chemistry linker, a polynucleotide (e.g., poly(T), (T)_n, poly(G), (G)_n, (GGGS)_n, where n is any useful integer, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, etc.), and/or a carbocyclic ring (e.g., an aromatic ring, such as a phenyl group).

Each primer set selected preferably consists essentially of or consists of the sequences in the SEQ IDs. Preferably, Set 12 further comprises SEQ ID No. 3 and/or SEQ ID No. 4; Set 10 further comprises SEQ ID No. 13 and/or SEQ ID No. 14; and Set 20 further comprises SEQ ID No. 23 and/or SEQ ID No. 24. Advantageously, the loop primers provide a boost to the amplification process to increase the sensitivity of the primer sets when used.

Preferably, a sequence of any one of the first linker, the second linker, the third linker, the fourth linker, the fifth linker and the sixth linker has minimal complementarity to the SEQ ID numbers (Nos.) in the respective set.

- 5 Preferably, a sequence of any one of the first linker, the second linker, the third linker, the fourth linker, the fifth linker and the sixth linker is TTTT. More preferably, the primer set is selected from the group consisting of: Set 12: SEQ ID Nos. 1 to 6; Set 10: 11 to 16; and Set 20: 21 to 26; or a combination of any two or more of these primer sets.
- 10 By "primer", it is meant to refer to a nucleic acid molecule composed of single-stranded oligonucleotides, and nucleotides may be added and extended by covalent bonds at the 3' terminus of the primer during a reaction of nucleic acid amplification or synthesis using a polymerase. The primer set according to the present invention is used for amplification of nucleic acids.
- 15 As used interchangeably herein, the terms "nucleic acid (molecule(s))", "oligonucleotide(s)", and "polynucleotide(s)" include RNA or DNA (either single or double stranded, coding, complementary or antisense), or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form (although each of the above species
- 20 may be particularly specified). The term "nucleotide" is used herein as an adjective to describe molecules comprising RNA, DNA, or RNA/DNA hybrid sequences of any length in single-stranded or duplex form. More precisely, the expression "nucleotide sequence" encompasses the nucleic material itself and is thus not restricted to the sequence information (i.e. the succession of letters chosen among the four base letters) that biochemically
- 25 characterizes a specific DNA or RNA molecule. The term "nucleotide" is also used herein as a noun to refer to individual nucleotides or varieties of nucleotides, meaning a molecule, or individual unit in a larger nucleic acid molecule, comprising a purine or pyrimidine, a ribose or deoxyribose sugar moiety, and a phosphate group, or phosphodiester linkage in the case of nucleotides within an oligonucleotide or polynucleotide. The term "nucleotide" is
- 30 also used herein to encompass "modified nucleotides" which comprise at least one modification such as (a) an alternative linking group, (b) an analogous form of purine, (c) an analogous form of pyrimidine, or (d) an analogous sugar. For examples of analogous linking groups, purine, pyrimidines, and sugars, see, for example, PCT publication No. WO 95/04064, which disclosure is hereby incorporated by reference in its entirety. Preferred

modifications of the present invention include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v) ybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, and 2,6-diaminopurine. The polynucleotide sequences of the invention may be prepared by any known method, including synthetic, recombinant, ex vivo generation, or a combination thereof, as well as utilizing any purification methods known in the art. Methylenemethylimino linked oligonucleosides as well as mixed backbone compounds having, may be prepared as described in U.S. Pat. Nos. 5,378,825; 5,386,023; 5,489,677; 5,602,240; and 5,610,289, which disclosures are hereby incorporated by reference in their entireties. Formacetal and thioformacetal linked oligonucleosides may be prepared as described in U.S. Pat. Nos. 5,264,562 and 5,264,564, which disclosures are hereby incorporated by reference in their entireties. Ethylene oxide linked oligonucleosides may be prepared as described in U.S. Pat. No. 5,223,618, which disclosure is hereby incorporated by reference in its entirety. Phosphinate oligonucleotides may be prepared as described in U.S. Pat. No. 5,508,270, which disclosure is hereby incorporated by reference in its entirety. Alkyl phosphonate oligonucleotides may be prepared as described in U.S. Pat. No. 4,469,863, which disclosure is hereby incorporated by reference in its entirety. 3'-Deoxy-3'-methylene phosphonate oligonucleotides may be prepared as described in U.S. Pat. Nos. 5,610,289 or 5,625,050 which disclosures are hereby incorporated by reference in their entireties. Phosphoramidite oligonucleotides may be prepared as described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878 which disclosures are hereby incorporated by reference in their entireties. Alkylphosphonothioate oligonucleotides may be prepared as described in published PCT applications WO 94/17093 and WO 94/02499 which disclosures are hereby incorporated by reference in their entireties. 3'-Deoxy-3'-amino phosphoramidate oligonucleotides may be prepared as described in U.S. Pat. No. 5,476,925, which disclosure is hereby incorporated by reference in its entirety. Phosphotriester oligonucleotides may be

prepared as described in U.S. Pat. No. 5,023,243, which disclosure is hereby incorporated by reference in its entirety. Borano phosphate oligonucleotides may be prepared as described in U.S. Pat. Nos. 5,130,302 and 5,177,198 which disclosures are hereby incorporated by reference in their entireties.

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The terms "nucleoside" and "nucleotide" will include those moieties which contain not only the known purine and pyrimidine bases, but also other heterocyclic bases which have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Modified nucleosides or nucleotides can also include

10 modifications on the Sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or are functionalized as ethers, amines, or the like. The term "nucleotidic unit" is intended to encompass nucleosides and nucleotides.

By "nucleic acid", it is meant to refer to RNA or DNA molecules or analogs and derivatives thereof including one or more nucleotides of any form, including single- or double-stranded oligonucleotides or polyoligonucleotides. The nucleic acids of a SARS-CoV-2 virus to be

15 detected using the primer set according to the present invention may include RNA derived from the virus, for example, all or a part of the genome of the virus, or DNA corresponding thereto.

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By "attached", it is meant to refer to the two sequences to be joined by a chemical bond.

By "detect", it is meant to include determining whether the virus is present, have infected a subject, determining the prognosis of an infected subject, and determining recurrence after

25 infection treatment (for example, monitoring the status of the subject to provide information about the therapeutic efficacy).

These primer sequences are set out below.

Primer set 12 SEQ ID NOS:

SEQ ID No.	Primer	Sequence
1	F3	ATATGAGAGTTATACATTTTGGTGC
2	B3	CTTAGGGTCGTACATATCACT
3	LF	GTCTTAAACAGCTGTACCTGGT

4	LB	CAACTTTGATTGGTGATTGTGCA
5	FIP	GACAAGCAGCGTACCCGTAGTTTTGGTTCTGATAA AGGAGTTGC
6	BIP	AATGACTTTGTCTCTGATGCAGATTTTTTGAGATCC CATTTATTAGCTGTA
7	FIP binding region 1	GACAAGCAGCGTACCCGTAG
8	FIP binding region 2	TGGTTCTGATAAAGGAGTTGC
9	BIP binding region 1	AATGACTTTGTCTCTGATGCAGATT
10	BIP binding region 2	TGAGATCCCATTTATTAGCTGTA

Primer set 10 SEQ ID NOS:

SEQ ID No.	Primer	Sequence
11	F3	TGTTTCGCGCTACTGCAACGA
12	B3	GATAGAGAAAAGGGGCTTCAAGGCC
13	LF	GCAACGCCAACAATAAGCCATCCG
14	LB	GGGTGTTCACTTTGTTTGCAACTGC
15	FIP	TGGAAGCGCTCTGAAAAACAGCATTTTACCGATAC AAGCCTCACTCCCTT
16	BIP	AGAGATGGCAACTAGCACTCTCCATTTTACGAGCA AAAGGTGTGAGTAACTGT
17	FIP binding region 1	TGGAAGCGCTCTGAAAAACAGCA
18	FIP binding region 2	ACCGATACAAGCCTCACTCCCTT
19	BIP binding region 1	AGAGATGGCAACTAGCACTCTCCA
20	BIP binding region 2	ACGAGCAAAAGGTGTGAGTAACTGT

Primer set 20 SEQ ID NOS:

SEQ ID No.	Primer	Sequence
21	F3	GCCAATAGGTATTAACATCACTAG
22	B3	CCTTTTTCTACAGTGAAGGATT
23	LF	CCAACCTGAAGAAGAATCACCAG
24	LB	AGACTGTGCACTTGACCCTC
25	FIP	ACATAATAAGCTGCAGCACCAG <u>TTTT</u> GCTTTACAT AGAAGTTATTTGACTC
26	BIP	AAATGGAACCATTACAGATGCTGT <u>TTTT</u> ACGTACA CTTTGTTTCTGAGA
27	FIP binding region 1	ACATAATAAGCTGCAGCACCAG
28	FIP binding region 2	GCTTTACATAGAAGTTATTTGACTC
29	BIP binding region 1	AAATGGAACCATTACAGATGCTGT
30	BIP binding region 2	ACGTACACTTTGTTTCTGAGA

The intervening 'TTTT' sequence (SEQ ID No. 133) in each of the FIP and BIP primer set above and in Table 2 is underlined and links the respective binding regions immediately preceding and following each underlined intervening 'TTTT' sequence.

The intervening 'TTTT' sequence in the FIP and BIP primers may be replaced by other nucleotides while retaining their effectiveness. The FIP binding regions 1 and 2 are joined by a linker (or intervening sequence), wherein the linker may be any nucleotide sequence of length 1 to 100 except for the reverse complement of the nucleotide sequence between the two binding regions in the SARS-CoV-2 RNA. The BIP binding regions 1 and 2 are joined by a linker (or intervening sequence), wherein the linker may be any nucleotide sequence of length 1 to 100 except for the reverse complement of the nucleotide sequence between the two binding regions in the SARS-CoV-2 RNA. In other words, if the SARS-CoV-2 RNA nucleotide sequence between the two regions binding to the FIP or BIP is CGGA, the linker in the FIP or BIP cannot be TCCG (the reverse complement of the natural sequence).

Each linker in the FIP and/or BIP primer for each set may contain 1 to 100 nucleotides. In an embodiment, the linker contains 1 to 75 nucleotides. In another embodiment, the linker contains 1 to 50 nucleotides. In another embodiment, the linker contains 1 to 40 nucleotides.

5 In another embodiment, the linker contains 1 to 30 nucleotides. In another embodiment, the linker contains 1 to 20 nucleotides. In another embodiment, the linker contains 1 to 10 nucleotides. In another embodiment, the linker contains 3 to 10 nucleotides. Preferably, the linker (intervening sequence) should have minimal complementarity to the binding regions in the FIP/BIP primer and the other LAMP primers to minimise primer dimerization.

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The primers of the invention have sequences that are substantially similar or identical to the sequences of the primers represented by SEQ ID Nos 1 to 30 above or have a complementarity of 70%, preferably a complementarity of 80%, more preferably a complementarity of 90%, most preferably a complementarity of 95%, 96%, 97%, 98%, 99%,
15 or 100%, with the primers represented by SEQ ID Nos 1 to 30.

The terms “percentage of sequence identity” and “percentage homology” are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison
20 window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to
25 yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Identity is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA,
30 CLUSTAL W, FASTDB [Pearson and Lipman, (1988), Proc. Natl. Acad. Sci. USA 85(8):2444-2448; Altschul et al., (1990), J. Mol. Biol. 215(3):403-410; Thompson et al. (1994), Nucleic Acids Res. 22(2):4673-4680; Higgins et al., (1996), Meth. Enzymol. 266:383-402; Altschul et al., (1993), Nature Genetics 3:266-272; Brutlag et al. (1990)

Comp. App. Biosci. 6:237-24], the disclosures of which are incorporated by reference in their entireties.

By "complementarity", it is meant to refer to a condition in which a primer or
5 oligonucleotides can bind sequence-specifically to target nucleic acids through the Watson-Crick base pairing under hybridisation, binding or annealing conditions, and includes partial complementarity, substantial complementarity and perfect complementarity. Substantial complementarity means that two strands of nucleic acid sequences are not completely complementary to each other, but are complementary enough to bind to target nucleic acids
10 so as to amplify the effect according to this invention, that is, amplify the target sequence of a SARS-CoV-2 virus' nucleic acid through a nucleic acid amplification process.

By "hybridisation", it is meant to refer to a reaction in which two strands of complementary nucleic acid molecules form a sequence-specific complex through hydrogen bonding.
15 Hybridisation may constitute a step of binding a primer to target nucleic acids or a template in a LAMP reaction (or any other nucleic acid amplification reaction) in which the primers according to this invention is used. The degree of hybridisation is affected by various factors, such as the degree of complementarity of any two nucleic acid molecules, melting temperatures (T_m) thereof, or the stringency of hybridisation. The hybridisation reaction
20 conditions may also be determined by considering these factors. The stringency of a hybridisation reaction is a condition that determines how easily any two nucleic acid molecules to be hybridized can bind to each other, and the stringency may be determined depending on complementarity, reaction temperatures, ionic strength and/or concentrations and types of common substances included in a hybridisation reaction solution (see J. Sambrook and D. W. Russell, Molecular Cloning: A Laboratory Manual, Cold Spring
25 Harbor Laboratory Press; 4th Ed., 2012; and F. M. Ausubel, Ed., Short Protocols in Molecular Biology, Wiley; 5th Ed, 2002).

Specific binding or hybridisation herein means that a particular nucleic acid molecule or
30 primer binds only to target nucleic acids without substantial binding to nucleic acids other than target nucleic acids. The primers according to this invention may specifically bind to target SARS-CoV-2 nucleic acids according to this invention or the complementary sequence thereof under high stringency conditions. In the case of template-based DNA

synthesis, it may be determined in consideration of the length of a primer sequence, buffer, nucleic acid type, pH, magnesium concentration, and reaction temperature.

5 The primers according to this invention are designed to specifically detect SARS-CoV-2, and specifically bind to each of target nucleic acids or complementary sequence thereof.

10 The design of primer sets is non-obvious, as evident by the high failure rates for efficient reaction (approximately 80% do not perform satisfactorily, when designed and evaluated by a PHOSITA). This may be seen in Figure 3 which shows that most primer sets do not show sensitivity at or below 100 copies of SARS-CoV-2 RNA / reaction. Comparison with the best-performing prior art indicates that this technology discloses a superior composition as quantified via successful detection of the SARS-CoV-2 RNA. The superior performance is further surprising because individually the primer sets do not perform better than the prior art, whereas the combination of primer sets outperforms the prior art (both individual sets
15 or primer sets in combination). These are shown in Figures 5 and 6 below. Figure 5 shows that individual primers do not show very high detection (y-axis, and the number above each bar), whereas in combination (Figure 6) the disclosed primer sets 12+20 outperforms the best-performing prior art As1e+N+E.

20 In an embodiment, the primer set comprises, or consists essentially of, or consists of, Set 12 and Set 20, with the loop primers being preferably included but optional. In another embodiment, the primer set comprises, or consists essentially of, or consists of, Set 12, Set 20 and Set 10, with the loop primers being preferably included but optional.

25 In various embodiments, the nucleic amplification analysis is LAMP or RT-LAMP.

In various embodiments, one or more primers of the SEQ ID in each primer set is or are labelled with a detectable moiety.

30 Examples of detectable labels on LAMP primers include:

- Moieties as indicated in the review:
<https://pubs.rsc.org/en/content/articlehtml/2020/ay/c9ay02246e>
- Fluorophore moiety conjugates

- Fluorophore and quencher moiety conjugates
(<https://www.nature.com/articles/s41598-018-23930-1>)
- Internal nucleotide barcode(s) contained within LAMP primer sequences
(<https://www.medrxiv.org/content/10.1101/2020.08.07.20161737v1.full.pdf>,
5 <https://www.nature.com/articles/s41587-021-00966-9>)
- Endonuclease restriction sites contained within LAMP primer sequences
<https://www.mdpi.com/1420-3049/21/1/111/htm>
- Nanoparticle (gold) conjugates
<https://www.frontiersin.org/articles/10.3389/fchem.2018.00090/full>
- 10 • Combinations of any of the above

In a second aspect of the invention, there is provided a method to detect a SARS-CoV-2 virus in a sample, the method comprising: (a) providing a primer set according to the first aspect of the invention; (b) carrying out a nucleic acid amplification reaction using the
15 sample and the primer set to produce a reaction product mixture; and (c) analysing the reaction product mixture to determine the presence of the SARS-CoV-2 virus in the sample.

The method may include analysing the results of the nucleic acid amplification reaction and comparing the reaction results of the samples with the reaction results of control samples to
20 determine whether the samples are infected with SARS-CoV-2 virus. At this time, when there is a change in the amount of the amplified products of the sample as compared with the control sample, the sample is judged to be infected with SARS-CoV-2 virus. With design and selection of the analysis method, the readout of the analysis results may directly provide the comparison results as further explained below.

25 In various embodiments, the nucleic acid amplification reaction is LAMP or RT-LAMP. In various embodiments, the sample is any one selected from the group consisting of whole blood, plasma, serum, lymph, urine, saliva, tears, faeces, sewage, and nasopharyngeal secretions.

30 The nucleic acid amplification analysis or reaction will include the use of suitable or typical components known to the skilled person. For example, the use of magnesium, dNTPs, a reaction buffer, and a DNA polymerase. The analysis will include incubating the reaction

mixture under DNA polymerase reactions conditions to produce a reaction product mixture that can then be detected. When RNA is used as a template in a LAMP analysis, a reverse transcriptase may be additionally added to the reactant of the LAMP analysis and RT-LAMP may be performed.

5

Magnesium may be used in the form of a salt, such as magnesium acetate, magnesium chloride, or magnesium sulfate. A sodium phosphate buffer, a potassium phosphate buffer, a Tris-HCl buffer, or a Tricine buffer may be used as a buffer solution.

- 10 Non-limiting examples of the DNA polymerase that may be used in a reaction include the *Bacillus stearothermophilus* (Bst) DNA polymerase, the *Bacillus subtilis* (Bsu) polymerase, the Phi29 polymerase, the *Thermus thermophilus* (Tth) DNA polymerase, the *Thermus aquaticus* (Taq) DNA polymerase, the *Thermococcus litoralis* DNA polymerase, the *Pyrococcus furiosus* (Pfu) DNA polymerase, the Vent polymerase, the Q5 polymerase, the
- 15 9° North polymerase, the *Bacillus caldoteanax* DNA polymerase, the PyroPhage 3173 polymerase, and variants thereof.

- Non-limiting examples of reverse transcriptases that may be used in a reaction include the RTx reverse transcriptase, the SuperScript reverse transcriptase, the moloney murine
- 20 leukemia virus (MMLV) reverse transcriptase, the avian myeloblastosis virus (AMV) reverse transcriptase, the Bst polymerase, and variants thereof.

- In addition, nucleotide analogs may be used in place of dNTPs in a reactant. The nucleotide analogs may be modified nucleotides or nucleotides that are not found in nature, and may
- 25 be polymerized either alone or in conjunction with natural nucleotides during nucleic acid synthesis. Specific examples of nucleotide analogues that may be polymerized through the Watson-Crick base pairing include substituted purines or pyrimidines, deazapurines, methylpurines, methylpyrimidines, aminapurines, aminopyrimidines, thiopurines, thiopyrimidines, indole, pyrrole, 7-deazaguanine, 7-methylguanine, hypoxanthine,
- 30 pseudocytosines, pseudoisocytosines, isocytosines, isoguanine, 2-thiopyrimidines, 4-thiothymine, 6-thioguanine, nitropyrroles, nitroindoles, and 4-methylindole, without being limited thereto. Nucleotides including substituted deoxyribose analogs include substituted or unsubstituted arabinose, substituted or unsubstituted xylose, and substituted or unsubstituted pyranose. Nucleotides including phosphate ester analogs include

alkylphosphonates, such as phosphorothioates, phosphorodithioates, phosphoramidates, phosphoroselenoates, phosphoranilothioates, phosphoraniladates, phosphoramidates, boron phosphates, phosphotriesters, and methylphosphonates.

- 5 The reactants may react at a temperature suitable for the activities of a DNA polymerase and a reverse transcriptase. The reaction temperature may be determined in consideration of a target nucleotide sequence and enzymes used in the reaction. For example, from 50 °C to 75 °C, from 60 °C to 70 °C, about 65 °C. The reactant is allowed to react for a time sufficient to amplify the target nucleic acids. Those skilled in the art will be able to determine the
- 10 reaction time in consideration of the reaction conditions, for example, within a time period of 15 to 60 minutes, but the reaction time may vary depending on the amount of the target nucleic acids contained in a sample. For example, the reaction time may be increased to increase sensitivity.
- 15 In various embodiments, a DNA polymerase and a reverse transcriptase may be used together. In addition, some enzymes exhibit both DNA polymerase and reverse transcriptase functions (e.g. Bst 3.0). Thus, a reverse transcription reaction and an amplification reaction may be performed in one reaction, thereby increasing convenience. In particular, when samples, such as nasal discharge and blood, are directly used without isolating nucleic acids,
- 20 RNA and DNA of the viruses may be present in these samples, so that efficient amplification is possible. In the RT-LAMP reaction according to this invention, since reverse transcribed DNA as well as DNA may be amplified, DNA and/or RNA of proviruses may be amplified.

The LAMP analysis according to this invention may be performed in a variety of analysis

25 formats, for example, in the form of a liquid phase reaction or with several components adsorbed to a solid substrate.

In various embodiments, the step of analysing the reaction product mixture is by measuring one or more of the following: colour change (colorimetric or photometric method), pH, ionic

30 concentrations, fluorescence, nucleotide sequences, amplification products, or turbidity of the reactants.

The amplified reaction product after amplification may be detected in various ways. For example, the colour change and turbidity change of a reaction solution depending on DNA

synthesis, fluorescence and/or electrophoresis may be used. The detected products are compared to the products of positive and/or negative control samples to quantitatively or qualitatively analyse virus infection.

- 5 In various embodiments, the amplified products may be detected by the colour change of a reaction solution depending on DNA synthesis. In this case, the reaction liquid may contain an appropriate indicator, and for example, phenol red, a pH indicator whose colour changes depending on the pH of the solution, or hydroxy naphthol blue (HNB), a dye whose colour changes depending on the concentration of magnesium ions in a reaction solution, may be
10 used. The colour of the product of the reaction is compared with that of the products of positive and/or negative control samples to quantitatively or qualitatively analyse virus infection. Especially, since detection is possible with eyes, convenience may be increased.

- In various embodiments, nucleic acids contained in an amplified product may be detected
15 by a direct or indirect method. In the direct method, a detectably labelled moiety or primer may be used (examples are described above), and it is possible to confirm whether target nucleic acids are amplified by detecting a signal emitted from the labelled primer, which is specifically bound to the target nucleic acids. The detection may be performed in real time. In the indirect detection method, a labelled probe capable of binding to the amplified nucleic
20 acids may be used. As used herein, a detectably labelled probe is a probe labelled with a substance capable of emitting a signal which may be detected by any suitable method, such as spectroscopic, optical, photochemical, biochemical, enzymatic, electrical, and/or immunochemical methods. Examples of the substances include fluorescence moieties, chemiluminescent moieties, bioluminescent moieties, magnetic particles, enzymes,
25 substrates, radioactivity, and chromophore materials.

- In various embodiments, labels for detection include compounds capable of generating or eliminating a detectable fluorescent, chemiluminescent, or bioluminescent signal, such as light emitting, light scattering, and light absorbing materials, without being limited thereto.
30 For example, labels described in a reference (Garman A., Non-Radioactive Labeling, Academic Press 1997) may be referred to. Examples of fluorescent materials include fluorescein (e.g., US Patent No. 6,020,481), rhodamine (e.g., US Patent No. 6,191,278), benzophenoxazine (e.g., US Patent No. 6,140,500), energy transfer fluorescent dyes containing donors and receptors (e.g., US Patent No. 5,945,526) and cyanide (e.g., WO1997-

45539), lissamin, phycoerythrin, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, FluorX (Amersham), Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, 6-FAM, fluorescein isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, SYPRO, TAMRA, tetramethylrhodamine, and/or Texas Red, without being limited thereto. Also, any fluorescence moiety capable of generating other detectable signals is included. Fluorescent dyes include 6-carboxyfluorescein, 2',4',1,4-tetrachlorofluorescein, and 2',4',5',7',1,4-hexachlorofluorescein, without being limited thereto. In one embodiment, SYBR-Green, 6-carboxyfluorescein ("FAM"), TET, ROX, VIC, or JOE is used as a fluorescent marker.

In various embodiments, a probe labelled with two fluorescent materials, a reporting fluorescent material and an erasing fluorescent material, may be used. In this case, fluorescent materials emitting a spectrum of wavelengths that can be distinguished from each other are used. In addition, compounds capable of enhancing and stabilizing binding of nucleic acids or affecting binding of nucleic acids may be used as a marker, and examples of the compounds include intercalators including ethidium bromide, SYTO 9, and SYBR-Green, minor groove binders, and crosslinkable functional groups, without being limited thereto. Also, a reference (Blackburn et al., eds. "DNA and RNA Structure" in Nucleic Acids in Chemistry and Biology (1996)) may be referred to.

In addition, when confirming whether non-specific amplification by non-specific binding of primers occurs, a non-specific nucleic acid binder, such as ethidium bromide or PicoGreen, may be added to a control group not containing a template, and an amplification reaction is performed. After the reaction, the amount of amplified products non-specifically synthesized may be determined.

In a third aspect of the invention, there is provided a composition for detecting a SARS-CoV-2 virus, comprising a primer set according to the first aspect of the invention; a DNA polymerase and a reverse transcriptase. An enzyme which has both DNA polymerase and reverse transcriptase activity may also be used and would be considered to be present as both the DNA polymerase and reverse transcriptase.

In a fourth aspect of the invention, there is provided a kit for detecting a SARS-CoV-2 virus, comprising a primer set according to the first aspect of the invention; a DNA polymerase; a reverse transcriptase; and a positive control. An enzyme which has both DNA polymerase and reverse transcriptase activity may also be used and would be considered to be present
5 as both the DNA polymerase and reverse transcriptase.

The kit may include a positive control, a negative control, and instructions for use. As a negative control, a sample not containing nucleic acids may be included, and as a positive control group, nucleic acids extracted from one or more detection targets or chemically
10 synthesised may be included.

The primer, kit, and composition according to this invention may be used to detect SARS-CoV-2 virus at a nucleic acid level in biological samples. That is, the primer, kit, and composition may be used in an amplification reaction for detecting SARS-CoV-2infection.
15 Results obtained from samples suspected of having SARS-CoV-2 infection are compared with the results of a control group or a reference group to determine the presence of the nucleic acids of SARS-CoV-2 virus. At this time, based on the increase of the nucleic acids of SARS-CoV-2 or the quantitative change of the nucleic acids of SARS-CoV-2, it is possible to determine whether SARS-CoV-2 infection occurs. In addition, in the case of
20 receiving treatment after SARS-CoV-2 infection, the effect of the treatment may be predicted through the above process.

This invention may also then relate to a method of detecting a SARS-CoV-2 virus in biological samples to provide information necessary for detection, diagnosis, or prognosis
25 of COVID-19.

The method of detecting a SARS-CoV-2 virus according to this invention may be performed using the primer set, the composition or kit.

30 ***RT-LAMP protocols***

To empirically evaluate the designed LAMP primer sets, RT-LAMP (reverse transcription-loop mediated isothermal amplification) reactions were ran with a colorimetric readout by using a commercially available LAMP reagent kit (New England Biolabs, #M1800).

Synthetic RNA fragments of the SARS-CoV-2 genomic sequence (Twist Bioscience, #102024) serve as inputs with known concentrations for the RT-LAMP reactions.

Briefly, the RT-LAMP reactions were set up as such. A 10X LAMP primer mix was
5 prepared in nuclease-free water:

LAMP Primer (100 μ M stock in dH ₂ O)	Volume (μ L)
F3	2
B3	2
FIP	16
BIP	16
LF	4
LB	4
Nuclease-free water	56
Total	100

LAMP reactions were then prepared in PCR tubes as described in the Table 1 below, with
nuclease-free water used instead of DNA/RNA/sample for No Template Control (NTC)
10 conditions.

Table 1: Volume of components used in the LAMP reaction

Component	Volume (μ L)
Warmstart Colorimetric LAMP 2X Master Mix (NEB M1800)	12.5
4M Guanidine HCl	0.25
Nuclease-free water	8.75
10X LAMP Primer mix	2.5
DNA/RNA/sample or water	1
Total	25

Since (RT-)LAMP is a highly sensitive procedure, the protocol includes the following measures: (i) keeping a clean work area, (ii) NTC reactions, (iii) not opening the reaction

vessel after LAMP is complete, and (iv) if opening the vessel is unavoidable, to designate a post-amplification workspace far from the pre-amplification workspace.

To the traditional LAMP protocols, a further modification was made, which has been helpful in generating consistently more reliable and sensitive results: Instead of the routine approach of making a master mix with Warmstart Colorimetric LAMP Master Mix, water, guanidine HCl, and 10X LAMP primer mix, 10X LAMP primer mix was added to the sample before addition of the master mix (containing Warmstart Colorimetric LAMP Master Mix, guanidine HCl, and water).

The reaction tubes were then sealed and incubated at 65°C for 30 minutes. When completed, the tubes were removed for visual observation. Positive reactions are indicated by a change in colour of the solution from pink to yellow, while reactions containing negative controls (NTC) should remain pink. Certain reactions may change from pink to orange suggesting that LAMP amplification reactions have not reached completion when the assay ended at t=30min.

Screening of Prior Art

LAMP primer sets targeting different regions of the SARS-CoV-2 RNA have been published in online pre-prints, white papers, or other forms of prior art. A panel of these prior LAMP primers were evaluated and found that they performed reasonably well (majority of replicate reactions showing positive colour changes at 100 copies/reaction of synthetic RNA controls) (Figure 2). These LAMP primer sets consist of the As1e set (Rabe and Cepko, medRxiv, 2020), the 'NEB' primer sets E1 and N2 (Zhang et al., BioRxiv, 2020), the Lamb et al. set targeting Orf1a (Lamb et al., medRxiv, 2020), the DETECTR primer sets E and N (Broughton et al., Nature Biotechnology, 2020), and the STOPCOVID primer set (Joung et al., 2020). From these, it may be observed that the As1e, NEB E1 and NEB N2 sets show >90% positive results when tested at 100 copies/reaction of synthetic RNA controls with >25 replicates carried out.

Testing the LAMP primer sets

In parallel, the 20 LAMP primer sets designed with the LAMP primer design pipeline (Figure 3) and the NO series of LAMP primer set candidates designed manually (Figure 4) were tested to determine their effectiveness. From the computationally designed set of 20, 3

primer set candidates (primer sets 10, 12 and 20) that showed a clear majority of positive colorimetric RT-LAMP results at 100 copies/reaction synthetic RNA controls were identified and used for further assessment. The NO series of LAMP primer sets that were designed manually (“by eye”) did not show any positive results against 1000 copies/reaction or lower of synthetic RNA controls (Figure 4), suggesting that designing LAMP primers ‘by eye’ is not as robust a strategy as designing using the computational design pipeline. Importantly, the poor performance of most of the LAMP primer sets indicates that designing and identifying well-performing LAMP primer sets is not trivial nor obvious, which correspondingly indicates that the effective LAMP primer sets 10, 12 and 20 are non-obvious and requires an inventive step.

The multiplex SARS-CoV-2 LAMP primers outperforms prior art

Limit of Detection (LoD) Determination for individual primer sets

The limit of detection (LoD) for these LAMP primer sets was further investigated. LoD is defined as the sample concentration at which at least 95% of reactions (e.g. 19 out of 20) show true positive results i.e. a clear colour change in the colorimetric RT-LAMP reactions from pink to orange/yellow. This was done by conducting colorimetric RT-LAMP reactions in ≥ 20 replicates with the LAMP primer sets – the prior art of primer sets As1e, NEB E1, NEB N2; and the primer sets 10, 12 and 20 – at titrated concentrations of synthetic RNA controls (100, 75, 50 copies/reaction etc). It was determined that the LoD for individual primer sets As1e, NEB E1 and NEB N2 (prior art) are 75, 100 and 75 copies/reaction of synthetic RNA controls respectively, while the LoD for the primer sets 10, 12 and 20 are >100 , 100 and >100 copies/reaction respectively (Figure 1).

Figure 1 shows the Limit of Detection (LoD) determination of the best-performing primer sets including the existing LAMP primer sets As1e, NEB E1 and N2; and the new LAMP primer sets 10, 12 and 20. All 6 primer sets were tested at 50, 75 and 100 copies/reaction of synthetic RNA controls at 40 replicates per primer set per concentration of controls. For each concentration of synthetic RNA controls assayed, all 6 primer sets were tested on the same day in replicates of 20, using the exact same physical set of diluted synthetic RNA controls for a more reliable comparison across primer sets. Each primer set was assayed at the NTC condition for a total of 20 replicates as well across multiple days.

False positive rate

Apart from determining sensitivity (LoD), another key metric to consider for any diagnostic assay is the false positive rate. All the results presented here were obtained from colorimetric RT-LAMP reactions that were carried out with the abovementioned measures to reduce the occurrence of false positives. None of the primer sets showed false positives in any of the
5 NTC condition replicates, totalling >20 NTC replicates per primer set over multiple days (Figure 1).

Multiplexing primers by combining primer sets in one reaction

Two or more LAMP primer sets that target different regions of the SARS-CoV-2 genome
10 may be combined within an individual LAMP reaction in order to boost detection sensitivity of the assay. Notably, the As1e, NEB E1 and NEB N2 primer sets were combined in a single reaction to increase the detection sensitivity with 92% of reactions showing a true positive result at a concentration of 25 RNA copies per reaction, in contrast to each constituent primer set detecting the same sample concentration at about half the rate when used individually
15 (50%, 45% and 51% respectively for As1e, NEB E1 and NEB N2). Similar results were obtained to what was previously reported for these prior art (Figure 6).

Since the primer sets target different regions of the SARS-CoV-2 RNA (set 10: Orf3a; set 12: Orf1ab 2'-O-ribose methyltransferase; set 20: S gene), it is reasoned that combining
20 them could increase detection sensitivity than what each individual primer set could achieve singly. Surprisingly, the combination was observed to significantly improved detection sensitivity with the combination of primer sets 12 and 20 and primer sets 10, 12 and 20 (Figure 6). Unexpectedly, primer sets 12 and 20 in multiplex outperforms the combined primer sets As1e, NEB E1 and NEB N2, with the former combination achieving 90%
25 positive reactions at 12.5 copies per reaction of synthetic RNA controls while the latter combination achieves 82.5% positive reactions at the same sample concentration levels. The primer sets 12 and 20 in combination provides a significant improvement over the best-performing prior art.

30 Notably, increasing the number of primer sets within each LAMP reaction did not increase the rate of false positives – no positive reactions were observed for the NTC conditions for any of the multiplexed primer set combinations (Figure 6), suggesting that the specificity of the assay was not compromised despite the increase in detection sensitivity.

While the current examples used a commercially available LAMP reaction kit for demonstration, other LAMP reaction mixes may be used in lieu of the commercially available kit.

5 Table 2: Sequences of the 20 primer sets

SEQ ID No.	PRIMER	SEQUENCE
Primer Set 1		
31	F3	GGACCTGAGCGCACCTGTTGT
32	B3	GCCGCATTAATCTTCAGTTCATCACCA
33	LF	ACAGGCATAAGTGTCTGAAGCAGTGG
34	LB	TGACTAGGTGTCTAGCTGTCCACG
35	FIP	TGCTTTGTAGGTTACCTGTAAAACCCCTTTTGT GATAGACGTGCCACATGCTTT
36	BIP	TGTCAAGTCCATGGTAATGCACATGTTTTGTCC AGTCAACACGCTTAACAAAGC
Primer Set 2		
37	F3	TGGTGTTACCCGTGAACTCATGCG
38	B3	TGGACATTCCCCATTGAAGGTGTCA
39	LF	GCCATCAGGGCCACAGAAGTTGTT
40	LB	AGGGGTGTATACTGCTGCCGTGA
41	FIP	GCACGTGCTAGAAGGTCTTTAATGCATTTTACG GAGGGGCATACACTCGCT
42	BIP	TGCACTTTGTCCGAACAACTGGATTTTCGTTCCG TGTACCAAGCAATTTTCATGC
Primer Set 3		
43	F3	AGCATGGCCTCTTATTGTAACAGCTTT
44	B3	ACCTACAAGGTGGTTCCAGTTCTGT
45	LF	TGTCGTAGTGCAACAGGACTAAGCT
46	LB	GGGAGGTAGGTTTGTACTTGCACTGT
47	FIP	TGTGTAGTACCGGCAGCACAAGACATTTTAGGG CCAATTCTGCTGTCAAATTACA
48	BIP	ACTGCTTGCACTGATGACAATGCGTTTTTCCATC ACTCTTAGGGAATCTAGCCCA
Primer Set 4		
49	F3	CCAGGTTGCTGTTCTTTATCAGGATGT
50	B3	TGCACCAAGTGACATAGTGTAGGCA
51	LF	CGCCAAGTAGGAGTAAGTTGATCTGCA

52	LB	ACCCATTGGTGCAGGTATATGCG
53	FIP	ACAGCCTGCACGTGTTTGAAAAACATTTTACTG CACAGAAGTCCCTGTTGCT
54	BIP	GGGGCTGAACATGTCAACAACACTCATTTTACGT GCCCCGCCGAGGAGAA
Primer Set 5		
55	F3	TGGTTACTTACCCCAAAATGCTGTTGT
56	B3	CGGAACCTTCTCCAACAACACCTGT
57	LF	TGGTATTCGGCAAGACTATGCTCAGGT
58	LB	TGGTTGCCATAACAAGTGTGCCT
59	FIP	TGCGACCACCCTTACGAAGAATGGTTTTGTCCA GCATGTCACAATTCAGAAGT
60	BIP	CCTTTGGAGGCTGTGTGTTCTCTTTTTGCGCTA GCACGTGGAACCCA
Primer Set 6		
61	F3	GGCTTGATGTGGCTCAGCTACTTCA
62	B3	GGCAGGTCCTTGATGTCACAGCG
63	LF	TGGATTGAATGACCACATGGAACGCG
64	LB	AGTGAATCGTAATCGGAGCTGTGA
65	FIP	TGGAGTGGCACGTTGAGAAGAATGTTTTTGCTT CTTTCAGACTGTTTGCGCG
66	BIP	GGCACTATTCTGACCAGACCGCTTCTTTTTGTC CAGCAATACGAAGATGTCCACG
Primer Set 7		
67	F3	TGCATTGCCTGAGACGACAGCA
68	B3	ACCAAAGCACTCACAGTGTCAACA
69	LF	GGTCGCCAATGTACACATAGTGCT
70	LB	AGGTCCAGACATGTTCTCGGAA
71	FIP	GTGCGTGGTGCAGGTAATTGAGCTTTTTGTTGTC AATGCCAGATTACGTGCT
72	BIP	TGCTAACTAAGGGCACACTAGAACCAGTTTTTC AGCAGGACAACGCCGACA
Primer Set 8		
73	F3	TGCTTTCCATGCAGGGTGCTGT
74	B3	TGCCCTCTTGTCCTCAGATCTAGCC
75	LF	CTGAGGCTATAGCTTGTAAGGTTGCC
76	LB	TGAATTTGACCGTGATGCAGCCA
77	FIP	AAAAGCTGCATATGATGGAAGGGAACTTTTAA CAAGCTTTGTGAAGAAATGCTGGAC

78	BIP	GCAGGCTGTTGCTAATGGTGATTCTGATTTTGG GTCATAGCTTGATCAGCCATCT
	Primer Set 9	
79	F3	GCTCGAACTGCACCTCATGGTCA
80	B3	TCAGTGCCAAGCTCGTCGCC
81	LF	ACCACTACGACCGTACTGAATGCC
82	LB	ACGGTAATAAAGGAGCTGGTGGCCA
83	FIP	TGAGGGACAAGGACACCAAGTGTTTTTGGTTG AGCTGGTAGCAGAACTCG
84	BIP	GCGAAATACCAAGTGGCTTACCGCATTTTCAAA TGACTTTAGATCGGCGCCG
	Primer Set 10	
11	F3	TGTTTCGCGCTACTGCAACGA
12	B3	GATAGAGAAAAGGGGCTTCAAGGCC
13	LF	GCAACGCCAACAATAAGCCATCCG
14	LB	GGGTGTTCACTTTGTTTGCAACTTGC
15	FIP	TGGAAGCGCTCTGAAAAACAGCATTTTACCGAT ACAAGCCTCACTCCCTT
16	BIP	AGAGATGGCAACTAGCACTCTCCATTTTACGAG CAAAAGGTGTGAGTAACTGT
17	FIP binding region 1	TGGAAGCGCTCTGAAAAACAGCA
18	FIP binding region 2	ACCGATACAAGCCTCACTCCCTT
19	BIP binding region 1	AGAGATGGCAACTAGCACTCTCCA
20	BIP binding region 2	ACGAGCAAAAGGTGTGAGTAACTGT
	Primer Set 11	
85	F3	TTACACTAGTAATCCTACCACATTC
86	B3	CCTTCATGTGAATTATGAGGTTT
87	LF	ACCTTAATAGTCCTCACTTCTCTCA
88	LB	ATGACATATGGACAACAGTTTGGT
89	FIP	GGAGGTTAATGTTGTCTACTGTTGTTTTACCT TTGACAATCTTAAGACA
90	BIP	CGCAAGTTGTGGACATGTCATTTTCAGCTCCATC CAAATAAGTTG
	Primer Set 12	
1	F3	ATATGAGAGTTATACATTTTGGTGC
2	B3	CTTAGGGTCGTACATATCACT
3	LF	GTCTTAAACAGCTGTACCTGGT
4	LB	CAACTTTGATTGGTGATTGTGCA

5	FIP	GACAAGCAGCGTACCCGTAGTTTTGGTTCTGAT AAAGGAGTTGC
6	BIP	AATGACTTTGTCTCTGATGCAGATTTTTTGAGA TCCCATTTATTAGCTGTA
7	FIP binding region 1	GACAAGCAGCGTACCCGTAG
8	FIP binding region 2	TGGTTCTGATAAAGGAGTTGC
9	BIP binding region 1	AATGACTTTGTCTCTGATGCAGATT
10	BIP binding region 2	TGAGATCCCATTTATTAGCTGTA
Primer Set 13		
91	F3	TACAGACACTGGTGTGTAAC
92	B3	CCGAAACGAATGAGTACATAA
93	LF	GATTGTGTGAATTTGGACATGTTCT
94	LB	GACGACTACTAGCGTGCCTT
95	FIP	TGGATTAACAACCTCCGGATGAACCTTTTAATAA AATTGTTGATGAGCCTGA
96	BIP	AATGGAACCAATTTATGATGAACCGTTTTTCGTA CTCATCAGCTTGTG
Primer Set 14		
97	F3	GGACTACTCCCACCCAAGAATAGCA
98	B3	AAAAGCTGCATATGATGGAAGGGAAC
99	LF	ACTCTGAGTTGTTGCAAACTGAGAGT
100	LB	AACAAGCTTTGTGAAGAAATGCTGGAC
101	FIP	TGTAACCTGGACACATTGAGCCCACATTTTGGGT GTTGGTGGCAAACCTTGT
102	BIP	TGCTTTCCATGCAGGGTGCTGTTTTCTGAGGCT ATAGCTTGTAAGGTTGCC
Primer Set 15		
103	F3	TGCACTTTGTCCGAACAACTGGA
104	B3	TCGCCCCGTCTGCCATGAAGT
105	LF	CGTTCCGTGTACCAAGCAATTTTCATGC
106	LB	TCTGTCTATCCAGTTGCGTCACCA
107	FIP	TGGACATTCCCCATTGAAGGTGTCATTTTAGGG GTGTATACTGCTGCCGTGA
108	BIP	GGGTTGAAAAGAAAAAGCTTGATGGCTTTTTGA GTTGAAAGGCACATTTGGTTGCA
Primer Set 16		
109	F3	TATGATTTGAGTGTTGTCAATGC
110	B3	TTTGTCTTTATGTGCTTTAAGCT
111	LF	GTGGTGCAGGTAATTGAGCAG

112	LB	GTTGTCCTGCTGAAATTGTTGAC
113	FIP	GTGTGCCCTTAGTTAGCAATGTTTTATGTGTAC ATTGGCGACC
114	BIP	GTTCTCGGAACTTGTCGGCTTTTAACCAAAGCA CTCACAGT
Primer Set 17		
115	F3	TTTGGCTTTGTGTGCTGACTCTATCA
116	B3	GCACCGCCTTTGAGTGTGAAGGT
117	LF	GGGGCTTTTAGAGGCATGAGTAGGC
118	LB	GGGCTTATGTTGCTCGAAATCAAAGAC
119	FIP	ACTTCCTCTGTTAACACTTCTGTGGGATTTTTG TCACGCACTCAAAGGGATTGT
120	BIP	GCTGTTGAAGCTCCATTGGTTGGTTTTTGTAC CATCATATTAGGTGCAAGGGC
Primer Set 18		
121	F3	ACAAAACATTCCCACCAACAGAGCC
122	B3	TGGTGGCTCTTTCAAGTCCTCCCT
123	LF	TGCTGTTTCTTCTGTCTCTGCGGT
124	LB	ACCACACAAGGCAGATGGGC
125	FIP	TGCAGCAGGAAGAAGAGTCACAGTTTTTGAAGA AGGCTGATGAACTCAAGCCT
126	BIP	GCAGTGCTGACTCAACTCAGGCCTTTTCGTAAA CGGAAAAGCGAAAACGTTT
Primer Set 19		
127	F3	GGGCTTATGTTGCTCGAAATCAAAGAC
128	B3	CCACTCATCTAAATCAATGCCCAGTGG
129	LF	TGGTGCACCGCCTTTGAGTGT
130	LB	TCGGTACAGAAGTAAATGAGTTCGCC
131	FIP	TGCACTTCTATCACAGTGTCATCACCATTTTGCC CTTGACCTAATATGATGGTAACA
132	BIP	TGAGAAGTGCTCTGCCTATACAGTTGATTTTGC AAAGTTTTTATGACAGCATCTGCC
Primer Set 20		
21	F3	GCCAATAGGTATTAACATCACTAG
22	B3	CCTTTTTCTACAGTGAAGGATTT
23	LF	CCAACCTGAAGAAGAATCACCAG
24	LB	AGACTGTGCACTTGACCCTC
25	FIP	ACATAATAAGCTGCAGCACCAGTTTTGCTTTAC ATAGAAGTTATTTGACTC

26	BIP	AAATGGAACCATTACAGATGCTGTTTTACGTA CACTTTGTTTCTGAGA
27	FIP binding region 1	ACATAATAAGCTGCAGCACCAG
28	FIP binding region 2	GCTTTACATAGAAGTTATTTGACTC
29	BIP binding region 1	AAATGGAACCATTACAGATGCTGT
30	BIP binding region 2	ACGTACACTTTGTTTCTGAGA

In a first embodiment, there is a primer set for a nucleic acid amplification analysis to detect a SARS-CoV-2 virus, wherein the primer set is selected from the group consisting of: SEQ ID Nos. 1 to 6, 9 to 14; and 17 to 22; or a combination of any two or more of these primer sets.

Preferably, the nucleic acid amplification analysis is LAMP or RT-LAMP.

Preferably, one or more primers of each primer set is or are labelled with a detectable moiety.

In a second embodiment, there is a method to detect a SARS-CoV-2 virus in a sample, the method comprising:

- (a) providing a primer set according to the first embodiment above;
- (b) carrying out a nucleic acid amplification reaction using the sample and the primer set to produce a reaction product mixture; and
- (c) detecting the reaction product mixture to determine the presence of the SARS-CoV-2 virus in the sample.

Preferably, the nucleic acid amplification reaction is LAMP or RT-LAMP.

Preferably, the sample is any one selected from the group consisting of whole blood, plasma, serum, lymph, urine, saliva, tears, faeces, sewage, and nasopharyngeal secretions.

Preferably, the step of analysing results of the LAMP reaction is performed by measuring one or more of colour change, pH, ionic concentrations, fluorescence, nucleotide sequences, amplification products, or turbidity of the reactants.

in a third embodiment, there is a composition for detecting a SARS-CoV-2 virus, comprising a primer set according to the first embodiment above.

In a fourth embodiment, there is a kit for detecting a SARS-CoV-2 virus, comprising a
5 primer set according to the first embodiment above.

Whilst there has been described in the foregoing description preferred embodiments of the present invention, it will be understood by those skilled in the technology concerned that many variations or modifications in details of design or construction may be made without
10 departing from the present invention.

CLAIMS

1. A primer set for a nucleic acid amplification analysis to detect a SARS-CoV-2 virus, the primer set is selected from the group consisting of:
- 5 (a) Set 12: SEQ ID No. 1, SEQ ID NO. 2, SEQ ID No. 7 attached to SEQ ID No. 8 by a first linker, and SEQ ID No. 9 attached to SEQ ID No. 10 by a second linker, wherein the first linker and second linker each independently has 1 to 100 nucleotides, wherein the first linker does not consist of a first sequence complementary to a first nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 7 and SEQ ID
- 10 No. 8, wherein the second linker does not consist of a second sequence complementary to a second nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 9 and SEQ ID No. 10;
- 15 (b) Set 10: SEQ ID No. 11, SEQ ID NO. 12, SEQ ID No. 17 attached to SEQ ID No. 18 by a third linker, and SEQ ID No. 19 attached to SEQ ID No. 20 by a fourth linker, wherein the third linker and fourth linker each independently has 1 to 100 nucleotides, wherein the third linker does not consist of a third sequence complementary to a third nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 17 and
- 20 SEQ ID No. 18, wherein the fourth linker does not consist of a fourth sequence complementary to a fourth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 19 and SEQ ID No. 20;
- 25 (c) Set 20: SEQ ID No. 21, SEQ ID NO. 22, SEQ ID No. 27 attached to SEQ ID No. 28 by a fifth linker, and SEQ ID No. 29 attached to SEQ ID No. 30 by a sixth linker, wherein the fifth linker and sixth linker each independently has 1 to 100 nucleotides, wherein the fifth linker does not consist of a fifth sequence complementary to a fifth nucleotide sequence between the two binding regions in the SARS-CoV-2 virus that binds to SEQ ID No. 27 and SEQ ID No.
- 30 28, wherein the sixth linker does not consist of a sixth sequence complementary to a sixth nucleotide sequence between the two binding

regions in the SARS-CoV-2 virus that binds to SEQ ID No. 29 and SEQ ID No. 30; and

(d) any combinations of each set above.

- 5 2. The primer set according to claim 1 wherein Set 12 further comprises SEQ ID No. 3 and/or SEQ ID No. 4; Set 10 further comprises SEQ ID No. 13 and/or SEQ ID No. 14; and Set 20 further comprises SEQ ID No. 23 and/or SEQ ID No. 24.
- 10 3. The primer set according to any one of claims 1 or 2 wherein a sequence of any one of the first linker, the second linker, the third linker, the fourth linker, the fifth linker and the sixth linker has minimal complimentary to the SEQ ID Nos. in the respective set.
- 15 4. The primer set according to any one of claims 1 to 3 wherein a sequence of any one of the first linker, the second linker, the third linker, the fourth linker, the fifth linker and the sixth linker is TTTT.
- 20 5. The primer set according to any one of claims 1 to 4 comprising Set 12 and Set 20.
- 20 6. The primer set according to claim 5 further comprising Set 10.
- 25 7. The primer set according to any one of claims 1 to 6, wherein the nucleic acid amplification analysis is LAMP or RT-LAMP.
- 25 8. The primer set according to any one of claims 1 to 7, wherein one or more primers of the SEQ ID in each primer set is or are labelled with a detectable moiety.
- 30 9. A method to detect a SARS-CoV-2 virus in a sample, the method comprising:
 - (a) providing a primer set according to any one of claims 1 to 8;
 - (b) carrying out a nucleic acid amplification reaction using the sample and the primer set to produce a reaction product mixture; and
 - (c) analysing the reaction product mixture to determine the presence of the SARS-CoV-2 virus in the sample.

10. The method according to claim 9, wherein the nucleic acid amplification reaction is LAMP or RT-LAMP.
11. The method according to any one of claims 9 or 10, wherein the sample is any one
5 selected from the group consisting of whole blood, plasma, serum, lymph, urine, saliva, tears, faeces, sewage, and nasopharyngeal secretions.
12. The method according to any one of claims 9 to 11, wherein the step of analysing
10 the reaction product mixture is by measuring one or more of the following: colour change, pH, ionic concentrations, fluorescence, nucleotide sequences, amplification products, or turbidity of the reactants.
13. A composition for detecting a SARS-CoV-2 virus, comprising a primer set according to any one of claims 1 to 8; a DNA polymerase; and a reverse transcriptase.
- 15 14. A kit for detecting a SARS-CoV-2 virus, comprising a primer set according to any one of claims 1 to 8; a DNA polymerase; a reverse transcriptase; and a positive control.

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