Intrinsic signal amplification by type III CRISPR-Cas systems provides a sequence-specific SARS-CoV-2 diagnostic

Highlights

- Sequence-specific recognition of RNA by CRISPR Csm complex activates Cas10
- Cas10 polymerizes ATP to make cyclic oligonucleotides, pyrophosphates, and protons
- Cas10’s rapidly amplified products are detectable in 1–30 min
- RT-LAMP can be coupled to T7-Csm to rapidly and sensitively detect SARS-CoV-2 RNA
Intrinsic signal amplification by type III CRISPR-Cas systems provides a sequence-specific SARS-CoV-2 diagnostic

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SUMMARY

There is an urgent need for inexpensive new technologies that enable fast, reliable, and scalable detection of viruses. Here, we repurpose the type III CRISPR-Cas system for sensitive and sequence-specific detection of SARS-CoV-2. RNA recognition by the type III CRISPR complex triggers Cas10-mediated polymerase activity, which simultaneously generates pyrophosphates, protons, and cyclic oligonucleotides. We show that all three Cas10-polymerase products are detectable using colorimetric or fluorometric readouts. We design ten guide RNAs that target conserved regions of SARS-CoV-2 genomes. Multiplexing improves the sensitivity of amplification-free RNA detection from 10^7 copies/μL for a single guide RNA to 10^6 copies/μL for ten guides. To decrease the limit of detection to levels that are clinically relevant, we developed a two-pot reaction consisting of RT-LAMP followed by T7-transcription and type III CRISPR-based detection. The two-pot reaction has a sensitivity of 200 copies/μL and is completed using patient samples in less than 30 min.

INTRODUCTION

Frequent tests and quick results are critical for limiting the spread of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) and ending the current coronavirus disease 2019 (COVID-19) pandemic.1,2 qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction) has been the gold standard for viral diagnostics, but this method is slow and requires sophisticated equipment that is expensive to purchase and operate. A national survey of individuals tested using nasal swab-based qRT-PCR found that the average wait times for results was 4.1 days, with 10% of tests taking 310 days.3 Thus, there is an urgent need for inexpensive new technologies that enable fast, reliable, and scalable detection of viruses.

Recently, loop-mediated isothermal amplification (LAMP)4 was developed as a sensitive (1–100 copies/μL) point-of-care diagnostic.5,6 However, LAMP and reverse-transcription LAMP (RT-LAMP) are prone to generating mis-primed or template-independent amplicons that cause false positive readings if they are not coupled to sequence-specific verification of the amplicon.7–12 For example, the POP7-targeting primers used in the DETECTR CRISPR-based diagnostic lead to excessive non-specific amplification; however, the sequence-specific verification of the RT-LAMP amplicon by Cas12 yields sensitive and specific detection of POP7 RNA.12,13 Therefore, platforms that can further verify nucleic acid sequences increase the utility of LAMP and RT-LAMP applications. Type V (Cas12-based) and type VI (Cas13-based) CRISPR systems have been coupled to LAMP or RPA (recombinase polymerase amplification) for sensitive and reliable detection of viral nucleic acids.13–18 Following isothermal amplification, the RNA-guided Cas12 or Cas13 proteins bind to the amplified target and trigger a non-sequence-specific nuclease activity that cleaves a fluorophore and quencher-labeled DNA or RNA.14,15,18 Cleavage of the tether results in an increase in fluorescence that can be detected in 45 min. Furthermore, the collateral nuclease activity of Cas13 has previously been used to cleave and mature an RNA oligo into a linear pseudo-ligand for the RNase Csm6, resulting in a 3.5-fold increase signal sensitivity.14 While Cas12 and Cas13 detection methods have been optimized over several iterations to be compatible with the isothermal amplification of viral RNA, the ultimate goal is to develop CRISPR-based technologies that are sensitive enough to detect the viral RNA directly, without prior amplification. Recently, Fozouni et al.19 reported that type IV (Cas13a-based) CRISPR systems can be used for amplification-free detection of SARS-CoV-2 RNA in ~30 min and with the sensitivity of ~100 copies/μL.

Like the type VI (Cas13-based) systems, type III systems also target RNA.20–24 However, type III systems rely on a unique intrinsic signal amplification mechanism (Figure 1). Here, we
set out to determine whether intrinsic amplification by type III systems could be used to detect viral RNA without prior amplification. The proof-of-concept presented here demonstrates that the Casm complex from *Thermus thermophilus* (TtCsm) can be programmed to specifically recognize the SARS-CoV-2 genome. SARS-CoV-2, but not SARS-CoV-1 or a panel of other respiratory pathogens, activates the Cas10 polymerase, which generates ~1,000 cyclic nucleotides (e.g., cA4) after binding an RNA target.25–28 Like all polyamylases, nucleotide polymerization by Cas10 also generates protons (H+) and pyrophosphates (PPi). We demonstrate that each of these three products can be used to detect SARS-CoV-2 RNA using either colorimetric, fluorometric, or both methods simultaneously. The assay can be performed in 1–30 min, depending on the detection method and the concentration of the RNA. When coupled with RT-LAMP, the assay can be performed in <30 min and has a limit of detection of ~200 copies/μL. Collectively, this work indicates that the type III systems can be repurposed for fast and sensitive diagnostics.

**RESULTS**

**Sequence-specific activation of Cas10 polymerase yields three detectable products**

Sequence-specific recognition of RNA by type III CRISPR systems initiates a signaling cascade (Figure 1A).26–28 RNA binding by the *Thermus thermophilus* (TtCsm) complex triggers a conformational change that activates the Palm domain of the Cas10 subunit, which amplifies the RNA binding signal by converting ATP into ~1,000 cyclic oligoadenylates (e.g., cA4).25–28 We hypothesized that the intrinsic signal amplification unique to type III CRISPR systems would boost the sensitivity of direct RNA detection, while maintaining specificity. To test this hypothesis, we expressed and purified the type III-A CRISPR RNA (crRNA)-guided surveillance complex from TtCsm with a guide complementary to the N-gene of SARS-CoV-2 (crRNA_N1) (Figures 1B and S1; Table S1).

The Csm3 subunits, which form the “backbone” of the Csm complex, are nuclease that cleaves bound target RNA in 6-nucleotide increments.21,24,25 The cleaved RNA fragments dissociate and the Csm complex returns to the “inactive” state (i.e., no Cas10-polymerase activity) (Figure 1A).26,30 Thus, in the context of an immune response, the RNase activity of Csm3 moderates Cas10 polymerase activity to limit excess nuclease activation that may otherwise kill the cell.26,30,31 However, we reasoned that a Csm3 mutation that prevents target RNA degradation would have two related benefits as a diagnostic. First, an RNase-dead Csm complex is expected to stay bound to target RNA longer, which would sustain the Cas10 polymerase activity. Second, Csm3-mediated cleavage of the target RNA (e.g., SARS-CoV-2 RNA) would reduce the target RNA concentration over time and thus limit the sensitivity of the assay. Therefore, we mutated residues in the Csm3 subunit responsible for target RNA cleavage (D34A)24,29 and purified the RNase-dead complex (TtCsm-Csm3-D34A) (Figure S1). To measure the limit of detection (LoD), we added the mutant or wild-type Csm complex to a reaction containing the TtCsm6 nuclease, a fluorescent reporter (i.e., FAM-RNA-Iowa Black FQ), and a titration of RNA corresponding to the N-gene of either SARS-CoV-2 or SARS-CoV-1 (Figures 1C and S2; Tables S2 and S3). Single mismatches in the 5′ end of target RNAs have been shown to reduce cyclic oligoadenylate production by SepCsm (type III-A) by up to 30-fold as compared to the fully complementary target,25 while double-nucleotide mutations in the 3′ end of target RNAs ablated cyclic oligoadenylate production by SsoCsm (type III-D).31 Furthermore, single- and double-nucleotide complementarity between the crRNA 5′ handle and the target 3′ anti-tag significantly decreased the allosteric activation of Cas10 and cyclic oligoadenylate production by SthCsm (type III-A).31 Single mismatches in the target RNA have been shown to result in 10-fold lower amounts of cyclic oligoadenylate production by other Csm complexes.31 Using fluorometric detection, both the mutant and the wild-type Csm complex could detect the SARS-CoV-2 RNA at concentrations >10^8 copies per reaction, and neither complex cross-reacted with the SARS-CoV-1 RNA at the highest concentrations tested. The RNase-dead TtCsm complex was roughly 3-fold more sensitive than the wild type, with an LoD of ~10^7 copies per reaction.

In addition to fluorometric detection, we developed a colorimetric RNA detection method that uses a pH change that occurs during nucleotide polymerization (Figures 1D and S3). Specific recognition of SARS-CoV-2 by RNase-dead TtCsm complex activates Cas10. Cas10 polymerizes ATP,25–28 releasing one proton per incorporated nucleotide. Cas10-generated protons acidify the solution and change the color of a pH indicator (i.e., Phenol Red) from fuchsia through orange (10^10 RNA copies) to yellow (10^11 RNA copies). Similarly, we developed a visible fluorometric detection method that relies on the sequestration of metallic ions by pyrophosphate. The metal indicator calcein is initially quenched by bound Mn^2+ ions.33 In addition to the cyclic oligoadenylates and protons, Cas10 polymerase generates one pyrophosphate per ATP polymerized. Pyrophosphate forms an insoluble precipitate with Mn^2+, which unquenches calcein. Free calcein is then bound by excess Mg^2+, forming a fluorescent complex that can be seen by eye or with a UV lamp in <10 min (Figures 1E and S4).

**Csm-based direct detection of SARS-CoV-2 RNA in patient samples**

The LoD using crRNA_N1 is between 10^7 and 10^8 copies of IVT RNA per microliter, which is insufficient to be clinically relevant (Figures 1C and 2C). To identify other guides that may outperform or complement the activity of crRNA_N1, we aligned 45,641 SARS-CoV-2 genomes available from GISAID.34 These alignments were used to select guides based on four key criteria. First, each target sequence had to be >99% identical among the available SARS-CoV-2 genomes. Second, complementarity between the target and the crRNA was not allowed to extend beyond the spacer sequence (guide) and into repeat derived portions of the crRNA that have been shown to suppress Cas10 activity.27 Third, we targeted regions of SARS-CoV-2 that were different by at least 2 nt in SARS-CoV-1 and MERS-CoV. Fourth, the list of target sequences was pruned to remove guides with similarity to human mRNA sequences or common oral and respiratory pathogen sequences (E value <1,000). Finally, we focused on target sequences located 3′ of the ORF3a gene, which are
present on both the viral genome and subgenomic RNAs generated during infection. In total, we designed crRNAs targeting 10 different locations on the SARS-CoV-2 genome (Figures 2A and S1; Table S1). To determine how each of these guides perform, we measured sequence-specific detection of RNA using a fluorometric reporter assay (i.e., FAM-RNA-Iowa Black FQ) (Figure 2B). Most of the crRNAs provide similar sensitivity; however, crRNAN1 and crRNAN9 generated significantly more signal than the next best complex tested (p < 0.0001). We then tested crRNAN1 and crRNAN9 on RNA isolated from the nasal swab of an infected patient (Figure 2C). Both crRNAN1 and crRNAN9 generated significantly more signal than the next best complex tested (p < 0.0001). We then tested crRNAN1 and crRNAN9 on RNA isolated from the nasal swab of an infected patient (Figure 2C). Both crRNAN1 and crRNAN9 generated significantly more signal than the next best complex tested (p < 0.0001). We then tested crRNAN1 and crRNAN9 on RNA isolated from the nasal swab of an infected patient (Figure 2C). Both crRNAN1 and crRNAN9 generated significantly more signal than the next best complex tested (p < 0.0001). We then tested crRNAN1 and crRNAN9 on RNA isolated from the nasal swab of an infected patient (Figure 2C). Both crRNAN1 and crRNAN9 generated significantly more signal than the next best complex tested (p < 0.0001). We then tested crRNAN1 and crRNAN9 on RNA isolated from the nasal swab of an infected patient (Figure 2C). Both crRNAN1 and crRNAN9 generated significantly more signal than the next best complex tested (p < 0.0001).
transcription (T7) and detected by TtCsm (Figures 3A and S5; Table S4).

To confirm the specificity of TtCsm-based detection, we tested SARS-CoV-2 alongside a panel of eight other oral and respiratory pathogens, including coronaviruses SARS-CoV-1, Middle East respiratory syndrome coronavirus (MERS-CoV), human coronavirus HKU1, and human coronavirus NL63 (Figure 3B). These samples resulted in a background signal similar to the no-template control (NTC) (Figure 3B). In contrast, SARS-CoV-2 RNA results in a 4- to 5-fold increase in signal.

To determine the LoD of RT-LAMP-T7-Csm, we tested 20 replicates of 2-fold serial dilutions ranging from ~100-400 copies/μL SARS-CoV-2 RNA (Figure 3C). The LoD of RT-LAMP-T7-Csm is 198 copies/μL SARS-CoV-2 RNA (20/20 replicates), in an assay that relies on a 29-min RT-LAMP step, followed by a 1-min T7-Csm fluorometric detection reaction (Figure 3D; Table 1).

To further validate this method, we next tested RNA extracted from 56 nasopharyngeal swab samples taken from patients who had previously been tested using qRT-PCR. Of the 56 samples tested, 46 were positive for SARS-CoV-2 and 10 were negative by qRT-PCR (Figure 3E). Using two different crRNA guides, we demonstrate that the type III CRISPR system has a specificity (negative predictive agreement) of 100%, as well as a positive predictive agreement of 100% for nasopharyngeal swab samples with 100–200 copies/μL SARS-CoV-2 RNA as determined by qRT-PCR (Figures 3E and S5). Whole-genome sequencing revealed that three of the patient samples used here belonged to the B.1.1.7 lineage. These genome sequences have been deposited in GISAID.
Importantly, the B.1.1.7 variants were positively identified by RT-LAMP-T7-Csm with both N1 and N9 crRNA guides (Figure 3E; N1, red circles; N9, blue triangles).

DISCUSSION

Collectively, this work demonstrates that sequence-specific detection of viral RNA by the type III CRISPR-Cas complex triggers the synthesis of cA4, pyrophosphate, and protons, each of which are detectable within 1–30 min, using colorimetric or fluorometric methods (Figure 1). Coupling RT-LAMP to T7 transcription and Csm-based detection creates a rapid (<30 min) testing protocol with attomolar sensitivity and high specificity. Moreover, we show that this protocol is capable of detecting the B.1.1.7 variant by both crRNAα1 and crRNAα9. Data are shown as fold change in fluorescence as compared to NTC reaction. Single technical replicates are shown for qRT-PCR and RT-LAMP-T7-Csm experiments with either N1 or N9 crRNAs.

While the LoD for RT-LAMP-T7-Csm is 1–1.5 orders of magnitude higher than FDA Emergency Use Authorization (EUA)-approved DETECTR and SHERLOCK assays (20 and 6.75 copies/μL, respectively), the time to result is ~30% faster, which would translate to a higher throughput of samples analyzed per unit of machine run time. Sensitivity was strategically sacrificed for time in this proof-of-concept application for optimal detection of infectious SARS-CoV-2 patients, which requires an LoD of ~1,000 copies/μL.1,2,35 However, an increase in the length of the RT-LAMP step (i.e., to 40 min, as is used in SHERLOCK), would likely increase the sensitivity. Additional modifications that could further increase sensitivity include the optimization of RT-LAMP primer sets, a screen for more active type III Csm or Cmr surveillance complexes, or a screen for CARF-fusion effectors that do not cleave their cyclic oligoadenylate ligands.39 Alternatively, more sensitive methods of measuring Cas10-generated protons, such as ion-sensitive field-effector transistors used in label-free sequencing by synthesis technologies, could decrease the LoD. Type V (Cas12-based) and type VI (Cas13-based) CRISPR-based diagnostics hinge completely on their collateral nucleolase
activity.13–17,40 The Cas10 subunits of Csm complexes possess an analogous nuclease activity triggered upon target binding.21,29,41–43 However, here, we focused on the NTP polymerase activity of the activated Csm complex, which enables readouts based on several different chemistries (Figure 1). Furthermore, there is a rich resource of naturally occurring downstream effector proteins that have evolved to be activated by the Cas10 polymerized NTP products, which possess a wide range of enzymatic activities. These effectors include RNases, DNases, putative proteases, nitrilases, adenosine deaminases, and adenylyl cyclases.44 Future efforts are aimed at incorporating complementary effectors with the goal of reducing the LoD for the direct detection of RNA in clinical samples.

Limitations of the study
This study demonstrates a proof-of-concept for a sensitive, specific, and rapid diagnostic based on the type III CRISPR system. The current implementation requires pre-amplification using RT-LAMP, followed by T7-transcription and type III detection (T3D). Clinical implementation of this approach will benefit from changes that eliminate the need for RT-LAMP and T7 amplification, improvements that limit sample handling, as well as a better understanding of the base pairing requirements that activate the Cas10 polymerase.16,19,26,30,32 Improvements will benefit from screening additional guides, testing other type III complexes for accelerated polymerase activity, and incorporating other ancillary nucleases or other effectors in a way that boosts the sensitivity and reduces the time to result.

STAR+METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Nucleic acid preparation
  - Plasmids

Table 1. Comparison of clinical parameters of RT-LAMP-T7-Csm and FDA EUA-approved protocols of DETECTR and SHERLOCK diagnostics27,38

| Test        | Time to result (min) | LoD (copies/μL) | Positive agreement (%) | Negative agreement (%) |
|-------------|----------------------|-----------------|------------------------|------------------------|
| DETECTR     | 45                   | 20              | 95                     | 95                     |
| SHERLOCK50  | 6.75                 | 100             | 100                    | 100                    |
| RT-LAMP-T7-Csm | 30              | 200             | 100                    | 74                     |

EUA, Emergency Use Authorization; FDA, US Food and Drug Administration; LoD, limit of detection.

o Protein purifications
o Type III CRISPR-based RNA detection
o Colorimetric CRISPR-Csm based detection
o Visible fluorometric CRISPR-Csm based detection
o RT-LAMP-T7-Csm
o Human clinical sample collection and preparation
o RT-qPCR
o Bioinformatic design of TtCsm crRNA guides targeting SARS-CoV-2
o Sequencing of SARS-CoV-2 RNA isolated from patient samples

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100319.

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AUTHOR CONTRIBUTIONS

B.W., A.S.-F., A. Nemudraia, and A. Nemudryi conceived the experimental plans. A.S.-F. and R.A.W. conducted the fluorometric and colorimetric Csm-based RNA detection. A.S.-F. conducted the visual fluorometric Csm-based RNA detection. L.N.H., P.K., A.G., and R.A.W. purified the proteins. A. Nemudraia and A. Nemudryi performed the qRT-PCR. A.S.-F., A. Nemudraia, and A. Nemudryi performed the RT-LAMP-T7-Csm-based RNA detection. T.W. performed the bioinformatic analyses and designed the crRNA guides. C.C. performed sequencing and analysis of the SARS-CoV-2 genomes. D.T.S., J.F.H., and H.H.L. performed the RNA extraction of patient nasopharyngeal swab samples under the guidance of M.A.J. and M.P.T. A.S.-F., T.W., A. Nemudraia, A. Nemudryi, and B.W. wrote the manuscript.

DECLARATION OF INTERESTS

B.W. is the founder of SurGene and VIRIS Detection Systems. B.W., A.S.-F., A. Nemudraia, and A. Nemudryi are inventors on patent applications related to CRISPR-Cas systems and applications thereof.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. One or more of the authors of this paper received support from a program designed to increase minority representation in science. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *E. coli*: Bl21 DE3 competent cells | NEB | Cat# C2527I |
| *E. coli*: DH5α competent cells | Thermo Fisher Scientific | Cat# 18265017 |
| **Biological samples** | | |
| SARS-CoV-2 | The National Institute of Standards and Technology | Cat# RGTM 10169 |
| SARS-CoV-1 | ATCC | Cat# VR-3280SD |
| MERS-CoV | ATCC | Cat# VR-3248SD |
| Human coronavirus HKU1 | ATCC | Cat# VR-3262SD |
| Influenza B | ATCC | Cat# VR-1885DQ |
| Human coronavirus NL63 | ATCC | Cat# VR-3263SD |
| Human respiratory syncytial virus | ATCC | Cat# VR-1580DQ |
| Pseudomonas aeruginosa | ATCC | Cat# 2783D-5 |
| Candida albicans | ATCC | Cat# 10231D-5 |
| Human Nasopharyngeal Swab RNA (Negative and Positive for SARS-CoV-2) | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| TCEP | Soltec | Cat# M115 |
| Protease inhibitor cocktail | Thermo Fisher Scientific | Cat# 1861278 |
| MEGAscript T7 transcription kit | Thermo Fisher Scientific | Cat# AMB13345 |
| SUMO protease | Wiedenheft lab | N/A |
| ATP | Thermo Fisher Scientific | Cat# R0441 |
| Murine RNase Inhibitor | NEB | Cat# M0314 |
| Calcein | MP Biomedical | Cat# 02190167-CF |
| WarmStart Bst 2.0 | NEB | Cat# M0538L |
| WarmStart RTx Reverse Transcriptase | NEB | Cat# M0380L |
| Hi-T7 RNA Polymerase | NEB | Cat# M0658 |
| TaqPath 1-Step RT-qPCR Master Mix | Thermo Fisher Scientific | Cat# A15299 |
| SuperScript IV | Thermo Fisher Scientific | Cat# 18090010 |
| Cyclic tetraadenylate | Biolog | Cat# C 335-005 |
| Q5 DNA Polymerase | NEB | Cat# M0491L |
| NEBNext Ultra II End Repair/dA-Tailing Module | NEB | Cat# E7546 |
| Native Barcoding Expansion 1-12 | Oxford Nanopore | Cat# EXP-NBD104 |
| Native Barcoding Expansion 13-24 | Oxford Nanopore | Cat# EXP-NBD114 |
| Ligation sequencing kit | Oxford Nanopore | Cat# SQK-LSK109 |
| WarmStart Colorimetric LAMP Master Mix | NEB | Cat# M1800L |
| **Deposited data** | | |
| B.1.1.7 lineage SARS-CoV-2 genome sequence 1 (hCoV-19/USA/MT-BHDH-227/2021) | This paper | GISAID ID: EPI_ISL_1081321; GenBank: MW940884 |
| B.1.1.7 lineage SARS-CoV-2 genome sequence 2 (hCoV-19/USA/MT-BHDH-228/2021) | This paper | GISAID ID: EPI_ISL_1081322; GenBank: MW940885 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the leader contact, Blake Wiedenheft (bwiedenheft@gmail.com).

Materials availability
Plasmids generated in this study are available upon request.

Data and code availability
Three sequenced SARS-CoV-2 genomes have been uploaded to GISAID (IDs EPI_ISL_1081321, EPI_ISL_1081322, EPI-ISL_1081323) and GenBank: MW940884, MW940885, MW940886. Genome IDs are listed in the Key resources table.

The script used to design TtCsm guide RNAs specific to SARS-CoV-2 genomes is available at https://github.com/WiedenheftLab/Type-III_crRNA_Design.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Escherichia coli* DH5α (Thermo Fisher Scientific) cells were used to amplify plasmids used in this paper. *E. coli* BL21 DE3 (NEB) cells were used to express proteins used in this paper. *E. coli* were grown in LB (Lennox) media at either 37°C, or 16°C after induction of protein expression with 0.5 mM IPTG (isopropyl-β-D-thiogalactoside).
Nucleic acid preparation

Previously published LAMP primers (Eurofins) were designed to amplify the SARS-CoV-2 N-gene. Target SARS-CoV-2 and SARS-CoV-1 RNAs were in vitro transcribed with MEGAscript T7 (Thermo Fisher Scientific) from PCR products generated from pairs of synthesized overlapping DNA oligos or using SARS-CoV-2 genome as a template (Table S3) (Eurofins). Previously designed primer pools (IDT) were used for RT-PCR and sequencing of SARS-CoV-2 genomes (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html). Transcribed RNAs were purified by denaturing PAGE. Fluorescent reporter RNA A and fluorescent reporter RNA B purified by RNase-free HPLC (Table S2) (IDT). Purified genomes of viral, bacterial and fungal pathogens were used as is, or resuspended in 1x TE (10 mM Tris-HCl pH 7.5, 1 mM Ethylenediaminetetraacetic acid (EDTA)) to -1x10⁶ genomes/μL (Table S5).

Plasmids

Expression vectors for *Thermus thermophilus* type III-A csm1-csm5 genes, pCDF-5xT7-TtCsm were purchased from Addgene (plasmid # 128572). pCDF-5xT7-TtCsm was used as a template for site-directed mutagenesis to mutate the Csm3 residue D33 to alanine (D33A) to inactivate Csm3-mediated cleavage of target RNA (pCDF-5xT7-TtCsm(Csm3-D33A)). The CRISPR array in pACYC-TtCas6-4xcrRNA4.5 (Addgene plasmid # 127764) was replaced with a synthetic CRISPR array (GeneArt) containing five repeats and four identical spacers, designed to target the N-gene of SARS-CoV2 (i.e., pACYC-TtCas6-4xgCoV2N1). TtCas6 was PCR amplified from the pACYC-TtCas6-4xcrRNA4.5 plasmid and cloned between the Ncol and Xhol sites of pRSF-1b (MilliporeSigma) (pRSF-TtCas6). The CARF-HEPN nuclease TtCsm6 was expressed from pC0075 TtCsm6 His6-TwinStrep-SUMO-BsaI (Addgene plasmid # 115270). Expression and purification of the TtCsm complex was performed as previously described with minor modifications. Briefly, the crRNA plasmid (e.g., pACYC-TtCas6-4xgCoV2N1) was co-transformed with pRSF-TtCas6 and either pCDF-5xT7-TtCsm or pCDF-5xT7-TtCsm(Csm3-D33A) into *Escherichia coli* BL21(DE3) cells and grown in LB Broth (Lennox) (Thermo Fisher Scientific) at 37°C to an OD₆₀₀ of 0.5. Cultures were then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactoside) for expression overnight at 25°C. Cells were pelleted (3,000 x g for 25 mins at 4°C) and lysed via sonication in Lysis buffer (25 mM HEPES pH 7.5, 150 mM KCl, 10 mM imidazole, 1 mM TCEP, 0.01% Triton X-100, 5% glycerol, 1 mM PMSF). Lysate was clarified by centrifugation at 10,000 x g for 25 mins at 4°C. The lysate was then heat-treated at 55°C for 45 minutes and further clarified by centrifugation at 10,000 x g for 25 mins at 4°C. His-tagged Csm1 and TtCsm complex were bound to HisTrap HP resin (Cytiva) and washed with Wash buffer (50 mM HEPES pH 7.5, 150 mM KCl, 1 mM TCEP, 5% glycerol, 20 mM imidazole). Protein was eluted in Lysis buffer supplemented with 300 mM imidazole. Eluted protein was concentrated (100k MWCO Corning Spin-X concentrators) at 4°C before further purification over HiLoad Superdex 200 26/600 or Superose 6 Increase 10/300 GL size-exclusion columns (Cytiva) in 25 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM TCEP. Fractions containing the TtCsm complex were pooled, concentrated, aliquoted, flash frozen in liquid nitrogen, and stored at −80°C.

Expression and purification of TtCsm6 was performed as previously described with minor modifications. pTtCsm6 was transformed into *Escherichia coli* BL21(DE3) cells and grown in LB Broth (Lennox) (Thermo Fisher Scientific) at 37°C to an OD₆₀₀ of 0.5. Cultures were then incubated on ice for 1 hour, and then induced with 0.5 mM IPTG for expression overnight at 16°C. Cells were lysed via sonication in TtCsm6 Lysis buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM TCEP) and lysate was clarified by centrifugation at 10,000 x g for 25 mins at 4°C. The lysate was then heat-treated at 55°C for 45 minutes and clarified by centrifugation at 10,000 x g for 25 mins at 4°C. His6-TwinStrep-tagged TtCsm6 was bound to StrepTrap HP resin (Cytiva) and washed in TtCsm6 Lysis buffer. The protein was eluted with TtCsm6 Lysis buffer supplemented with 2.5 mM desthiobiotin and concentrated (10k MWCO Corning Spin-X concentrators) at 4°C. Affinity tags were removed from TtCsm6 using SUMO protease (100 μL of 2.5 mg/ml protease per 20 mg of TtCsm6 substrate) during dialysis against SUMO digest buffer (30 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM DTT, 0.15% [i-gal]) at 4°C overnight. Cleaved His6-TwinStrep tag and uncleaved His6-TwinStrep-TtCsm6 were removed by binding to HisTrap HP resin (Cytiva), and the flow-through was concentrated using Corning Spin-X concentrators at 4°C. Finally, TtCsm6 was purified using a HiLoad Superdex 200 26/600 size-exclusion column (Cytiva) in 20 mM Tris-HCl pH 7.5, 1 mM DTT, 400 mM monopotassium glutamate, 5% glycerol. Fractions containing TtCsm6 were pooled, concentrated, aliquoted, flash frozen in liquid nitrogen, and stored at −80°C.

To screen guide RNAs in a high throughput format, ten TtCsm complexes were first crudely purified. 8 mL cultures of *E. coli* BL21-DE3 cells transformed with pTtCsm and pT7-5xCRISPR-Cas6 were grown at 37°C and 250 RPM in LB media with selective antibiotics until they reached an OD₆₀₀ reading of 0.4. Protein expression was then induced with the addition of 0.5 mM IPTG to the media, and cells were grown overnight at 16°C. Cells were collected by centrifugation at 4000 RPM, and cell pellets were resuspended in 250 μL of Ni-NTA Equilibration buffer (PBS; 100mM sodium phosphate, 600mM sodium chloride, 0.05% Tween-20 Detergent, 30mM imidazole; pH 8.0). Resuspended cells were sonicated twice for twenty seconds, then clarified by centrifugation at 15,000 rpm for 20 minutes at −4°C to remove cellular debris. The lysate was then heat-treated at 55°C for 45 minutes, and re-clarified by centrifugation at 15,000 rpm, for 30 mins at 4°C. TtCsm was then purified using HisPur Ni-NTA magnetic beads (Thermo Fisher Scientific) according to the manufacturers recommendations, but with modified wash (25 mM HEPES pH 7.5, 150 mM NaCl, 0.05%
Tween-20, 1 mM TCEP) and equilibration (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) and elution buffers (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP, 300 mM Imidazole). TtCsm complex concentration was quantified on a Nanodrop (Thermo Fisher Scientific).

**Type III CRISPR-based RNA detection**

For experiments shown in Figure 1C, RNA was extracted from nasopharyngeal swabs derived from patients that tested negative for SARS-CoV-2 as determined by RT-qPCR. This RNA was used as is or spiked with in vitro transcribed SARS-CoV-2 or SARS-CoV-1 RNA. These RNA samples were mixed with 250 \( \mu \)M ATP, 500 nM fluorescent reporter RNA A, 500 nM TtCsm complex, and 2500 nM of TtCsm6 in reaction buffer (20 mM Tris-HCl pH 7.9, 200 mM Monopotassium glutamate, 10 mM Ammonium sulfate, 5 mM Magnesium sulfate and 1 mM TCEP) in a 30 \( \mu \)L reaction. Reactions were incubated at 60°C (CRISPR-Csm alone), and fluorescence was measured over time in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems), using the manufacturers default filter settings for FAM dye. Fluorescence measurements at an incubation time of 45 minutes are reported.

For experiments shown in Figure 2C, an RNA detection mixture was made containing either 25 nM TtCsm\(^{\text{Csm3-D34A}}\) N1, or 25 nM TtCsm\(^{\text{Csm3-D34A}}\) N9, or 2.5 nM each of ten complexes (TtCsm\(^{\text{Csm3-D34A}}\) N1, N3, N6, N7, N8, N9, N10, N11, N12 and I1), mixed with 250 \( \mu \)M ATP, 150 nM fluorescent reporter RNA B, 300 nM TtCsm6, in reaction buffer (20 mM Tris-HCl pH 7.8, 250 mM Monopotassium glutamate, 10 mM Ammonium sulfate, 5 mM Magnesium sulfate and 1 mM TCEP). 3 \( \mu \)L of RNA extracted from a patient nasopharyngeal swab with high SARS-CoV-2 viral load (~5x10\(^8\) copies/mL) was added to 27 \( \mu \)L of the above RNA detection mixture. Alternatively, RNA from this positive patient sample was first diluted 10– or 100–fold into RNA extracted from a patient negative for SARS-CoV-2 (CT > 40), and 3 \( \mu \)L of these dilutions was added to 27 \( \mu \)L of the above RNA detection reaction. Reactions were incubated at 60°C and fluorescence was measured every 10 s for up to 20 minutes, in a QuantStudio 3 Real-Time PCR system (ThermoFisher), using the manufacturers default filter settings for FAM dye.

**Colorimetric CRISPR-Csm based detection**

TtCsm\(^{\text{Csm3-D34A}}\) stocks were buffer exchanged into a low buffering capacity buffer (0.5 mM Tris-HCl pH 8.8, 50 mM Potassium chloride, 0.1% Tween-20). LAMP primers designed to amplify the SARS-CoV-2 \( \text{N} \)-gene,\(^{13}\) were added at an optimized final concentration of 0.2 \( \mu \)M each, 10 \( \mu \)M LoopF and LoopB, 1.6 \( \mu \)M BIP, 0.53 \( \mu \)M ATP, 500 nM fluorescent reporter RNA A, 500 nM TtCsm, 5.5 units of Hi-T7 RNA Polymerase (NEB), 150 nM fluorescent reporter RNA B, and 20 nM of either TtCsm\(^{\text{Csm3-D34A}}\) N1 or N9, in reaction buffer (40 mM Tris-HCl pH 7.5, 4 mM Magnesium chloride, 50 mM Sodium chloride, 2 mM spermidine, 1 mM DTT). Reactions were incubated at 65°C for up to 20 min and fluorescence kinetics was monitored in a QuantStudio 3 Real-Time PCR system (ThermoFisher) as described above.

LoD standards were prepared by diluting SARS-CoV-2 RNA into RNA extracted from COVID-19-negative patient nasopharyngeal swabs. Concentrations were determined with RT-qPCR using a standard curve generated from 10-fold dilution series (1x10\(^5\)-1x10\(^-2\)) of IVT fragment.
Human clinical sample collection and preparation

Nasopharyngeal swabs from patients that either tested negative or positive for SARS-CoV-2 were collected in viral transport media. RNA was extracted from all patient samples using QIAamp Viral RNA Mini Kit (QIAGEN).

RT-qPCR

RT-qPCR was performed using two primer pairs (N1 and N2) and probes from the 2019-nCoV CDC EUA Kit (IDT#10006606), SARS-CoV-2 in RNA-extracted, nasopharyngeal patient samples was detected and quantified using one-step RT-qPCR in ABI 7500 Fast Real-Time PCR System according to CDC guidelines and protocols (https://www.fda.gov/media/134922/download). In brief, 20 μL reactions included 8.5 μL of Nuclease-free Water, 1.5 μL of Primer and Probe mix (IDT, 10006713), 5 μL of TaqPath 1-Step RT-qPCR Master Mix (ThermoFisher, A15299) and 5 μL of the template. Nuclease-free water was used as negative template control (NTC). Amplification was performed as follows: 25°C for 2 min, 50°C for 15 min, 95°C for 2 min followed by 45 cycles of 95°C for 3 s and 55°C for 30 s. To quantify viral genome copy numbers in the samples, standard curves for N1 and N2 were generated using a dilution series of a SARS-CoV-2 synthetic RNA fragment (RTGM 10169, National Institute of Standards and Technology) spanning N gene with concentrations ranging from 10 to 10⁶ copies per μL. Three technical replicates were performed at each dilution. The NTC showed no amplification throughout the 45 cycles of qPCR.

Bioinformatic design of TtCsm crRNA guides targeting SARS-CoV-2

An alignment of 45,641 SARS-CoV-2 genomes was downloaded from the GISAID database (Global Initiative for Sharing All Influenza Data; https://www.gisaid.org/) on 6-23-2020. The alignment was scanned for conservation with a 40-nucleotide sliding window, and 40-nucleotide segments with high conservation were saved for downstream analysis. Next, four nucleotides flanking the above 40-nucleotide candidate viral target sequences were checked for base pairing to the first four nucleotides of the prospective 5’-crRNA handle (underlined; 5’-AUUGCGAC-3’), only candidates lacking handle complementarity were considered further. Candidate sites with less than two mismatches to SARS-CoV (NC_004718.3) and MERS-CoV (NC_019843.3) in the first 18 nucleotides of the target sequence were discarded. Next, candidate crRNAs targeting the above sites were screened for potential cross-reactivity with human mRNAs and a list of human pathogens and common respiratory flora downloaded from the FDA’s Emergency Use Authorization requirements (downloaded on 7-29-2020) using BLAST (E-value 1000). The remaining 6,229 crRNA sequences were then sorted by genomic location and only guides that were located 3’ of the SARS-CoV-2 ORF3a gene (positions 25,393 to 29,903) were considered further. Finally, 76 guides were selected from the remaining pool that had the greatest conservation among SARS-CoV-2 sequences and the largest number of mismatches to SARS-CoV and MERS-CoV sequences.

Sequencing of SARS-CoV-2 RNA isolated from patient samples

SARS-CoV-2 genomic RNA isolated from patient samples was sequenced as previously described. In brief, 10 μL of SARS-CoV-2 genomic RNA extracted from nasopharyngeal patient swabs was first reverse transcribed with SuperScript IV (ThermoFisher) according to the manufacturer’s instructions. The ARTIC Network protocol was followed to generate a sequence amplicon library covering the whole SARS-CoV-2 genome on Oxford Nanopore using a ligation sequencing kit (Oxford Nanopore, SQK-LSK109) (https://artic.network/nconv-2019/nconv2019-bioinformatics-sop.html). Two multiplex PCR reactions were performed with primer pools described in the ARTIC nCoV-2019 V3 Panel (Table S6), amplified with Q5 DNA Polymerase (NEB). The two resulting amplicon pools for each patient sample were then combined and used for library preparation. Samples were end repaired (NEB, E7546) and then barcoded using Native Barcoding Expansion Kits (Oxford Nanopore, EXP-NBD104 and EXP-NBD114). Barcoded samples were pooled together and then Nanopore adaptors were ligated.

The multiplexed library was loaded onto the MinION flowcell, and a total of 0.3 Gb of raw sequencing data was collected per patient sample. Raw Nanopore reads were base-called in high-accuracy mode (Oxford Nanopore, MinKNOW), and further analyzed using the ARTIC bioinformatic pipeline for COVID-19 (https://artic.network/nconv-2019/nconv2019-bioinformatics-sop.html). Consensus sequences were uploaded to GISAID (https://www.gisaid.org/), IDs: EPI-ISL_1081321, EPI-ISL_1081322, EPI-ISL_1081323. These three SARS-CoV-2 genome sequences were identified as members of the B.1.1.7 lineage by an automated lineage assigner (https://github.com/cov-lineages/pangolin).

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were performed in triplicate or duplicate and error is reported as ± 1 standard deviation. The merged datasets of replicates of fluorescence kinetics of direct Csm-based detection of SARS-CoV-2 RNA in patient samples was fit to a simple linear regression, in Prism 9 (Graphpad). The fitted slopes of SARS-CoV-2 RNA-containing patient samples were compared pairwise to the negative swab RNA control by an F-test, ****p < 0.0001.