

CREST, a Cas13-Based, Rugged, Equitable, Scalable Testing (CREST) for SARS-CoV-2 Detection in Patient Samples

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The COVID-19 pandemic has taken a devastating human toll worldwide. The development of impactful guidelines and measures for controlling the COVID-19 pandemic requires continuous and widespread testing of suspected cases and their contacts through accurate, accessible, and reliable methods for SARS-CoV-2 detection. Here we describe a CRISPR-Cas13-based method for the detection of SARS-CoV-2. The assay is called CREST (Cas13-based, rugged, equitable, scalable testing), and is specific, sensitive, and highly accessible. As such, CREST may provide a low-cost and dependable alternative for SARS-CoV-2 surveillance. © 2022 Wiley Periodicals LLC.

Basic Protocol: Cas13-based detection of SARS-CoV-2 genetic material using a real-time PCR detection system

Alternate Protocol: Cas13-based detection of SARS-CoV-2 genetic material using a fluorescence viewer

Support Protocol 1: LwaCas13a purification

Support Protocol 2: *In vitro* transcription of synthetic targets

Keywords: CRISPR • Cas13 • COVID-19 • diagnostic • SARS-CoV-2

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INTRODUCTION

With over 420 million cases diagnosed and 5.8 million deaths as of February 2022, SARS-CoV-2 has brought healthcare systems to the brink of collapse and disrupted the way of life of countless communities across the globe (Johns Hopkins University Coronavirus Resource Center; <https://coronavirus.jhu.edu>). The control of SARS-CoV-2 transmission is imperative to halt the COVID-19 pandemic. However, a better understanding of SARS-CoV-2 epidemiology remains an unmet need (Du et al., 2021). One way to address this issue relies on affordable, accurate, rapid, and robust methods for SARS-CoV-2

detection, whose accessibility is still a persistent challenge, especially in countries with minimal clinical laboratory infrastructure (Bhattacharjee et al., 2021).

SARS-CoV-2 possesses a positive-sense, single-stranded RNA genome of about 30,000 nucleotides (Hu, Guo, Zhou, & Shi, 2021). Diagnostic tests for SARS-CoV-2 involve the detection of viral antigens or viral RNA. The CDC recommends the use of reverse transcription quantitative polymerase chain reaction (RT-qPCR) as the “gold standard” for detection of viral RNA in human samples (Bhattacharjee et al., 2021). While RT-qPCR is reliable and accurate, it still requires specialized equipment, limiting reagents, and highly trained personnel, which may not be readily available in all regions (Bustin & Nolan, 2020; CDC, 2020; Vogels et al., 2020). Several research groups worldwide have devoted significant effort to developing reliable and accessible diagnostic tests for SARS-CoV-2 using minimal infrastructure and handling (Broughton et al., 2020; Zhang, Abudayyeh, 2020; Fozouni et al., 2021; Joung et al., 2020; Wyllie et al., 2020). Here we describe one such alternative: CREST (Cas13-based, Rugged, Equitable, Scalable Testing), a CRISPR-Cas13-based method that we recently developed for the qualitative detection of SARS-CoV-2 genome sequences in nasopharyngeal and oropharyngeal samples (Rauch et al., 2021). CREST exploits PCR’s robustness and reliability while harnessing the sensitivity and convenience of a coupled transcription-detection reaction. CREST uses specific forward and reverse primers to amplify the SARS-CoV-2 nucleocapsid (N) gene and the control cellular transcript RNase P. Following amplification, the PCR products are *in vitro* transcribed, and the resulting RNAs are the substrate for a CRISPR-based detection reaction in which the Cas13 enzyme is guided to the N or RNase P RNA products with specific small guide RNAs. When bound to its target, Cas13 catalyzes the non-specific cleavage of RNAs (Abudayyeh et al., 2017; Gootenberg et al., 2017), liberating a quencher from a fluorophore in a reporter probe. This target-specific recognition is thus detected by fluorescence visualization.

CREST is versatile and capitalizes on the strengths of RT-qPCR and CRISPR-based detection while addressing their shortcomings. From RNA sample to result, CREST can be run with no need for AC power or a dedicated facility, with minimal handling, in ~3 hr. It can also be used in specialized labs with manual or semi-automated processing, thus allowing scalable sample handling. Because the Cas13-dependent fluorescent reporter signal saturates even at low input levels, CREST offers the added advantage of a binary result interpretation.

Here we provide two protocols for the detection of SARS-CoV-2 viral RNA in extracted samples using CREST. The Basic Protocol relies on using a real-time PCR instrument to detect SARS-CoV-2 RNA in an extracted sample. An Alternate Protocol is provided that has been designed for testing in labs with limited infrastructure, as it relies on inexpensive thermocyclers and an endpoint fluorescent detection system that can be easily acquired and field-deployed. CREST offers a solution for places where access to professional laboratories is restricted or instances where a low-cost alternative for regular testing is necessary.

BASIC PROTOCOL

Cas13-BASED DETECTION OF SARS-CoV-2 GENETIC MATERIAL USING A REAL-TIME PCR DETECTION SYSTEM

Total RNA, the input for CREST, can be extracted using commercially available kits following manufacturer recommendations or with recently developed column-free protocols (Graham et al., 2021; Guruceaga, Sierra, Marino, Santin, & Mayor, 2020; Ponce-Rojas et al., 2020; Wozniak et al., 2020).

CREST comprises three main steps: (i) reverse transcription of viral genes from purified RNA, (ii) PCR-based amplification of a target region, and (iii) *in vitro* transcription coupled to Cas13-based detection of the region of interest (Fig. 1). Here we describe the

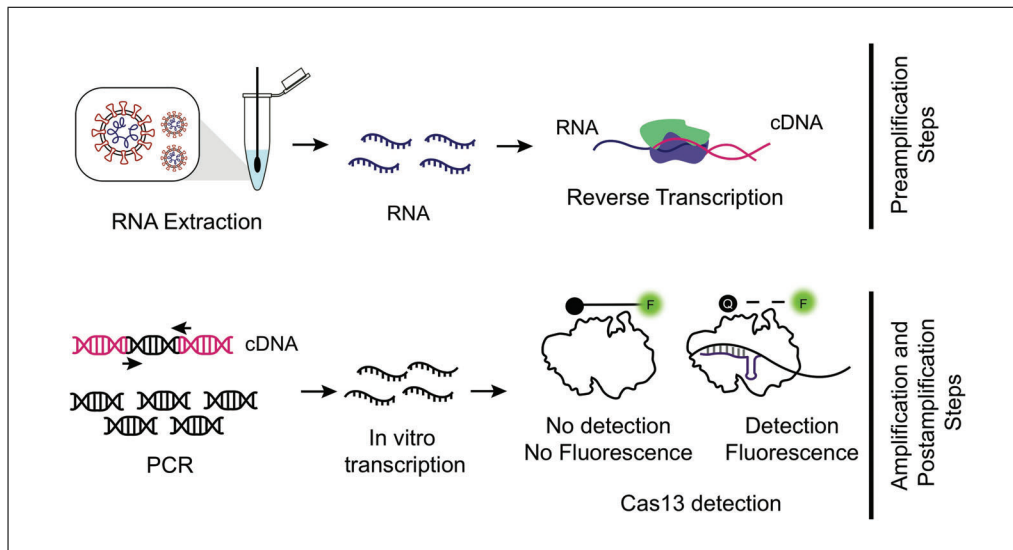


Figure 1 Experimental steps in CREST. RNA is extracted from nasopharyngeal (NP) or oropharyngeal (OP) swabs. The RNA is reverse transcribed, and the resulting DNA is amplified by the polymerase chain reaction (PCR) using primers for the SARS-CoV-2 N1 and N2 and the host RNaseP target regions. The PCR-amplified region of interest is transcribed *in vitro* and used as the template for detection by Cas13. The activation of Cas13 following target recognition by the guide RNA (gRNA) is measured using a fluorescent poly(U) cleavage reporter.

protocol for diagnosing the presence of the N1 or N2 genes of SARS-CoV-2 and the host RNaseP gene using a real-time PCR detection system. Each target gene is detected in a separate reaction, and therefore this protocol can be scaled up to test up to 30 samples in a 96-well format.

Materials

- RNase surface decontamination solution (RNase Away, RNase Zap)
- 100 μ M Gene-specific primers (Custom IDT order; See Table 1 for sequences)
- 5 \times Reaction Buffer for RT (ThermoFisher, EP0441)
- dNTPs at 10 mM each dNTP (NEB, N0447S or equivalent)
- RevertAid Reverse Transcriptase and 5 \times reaction buffer (Fisher, EP0442)
- Murine RNase Inhibitor (NEB, M0314L)
- Template RNA
- Nuclease-free water (NEB, B1500S or equivalent)
- Taq PCR 5 \times Master Mix (NEB, M0285)
- 10 \times Cleavage Buffer: 400 mM Tris·HCl, pH 7.5, containing 10 mM DTT
- rNTPs Mix (NEB, N0466S)
- CREST Fluorescent Cleavage Reporter (Table 1)
- LwaCas13a (See Support Protocol 1)
- T7 RNA polymerase (NEB, M0251S)
- Cas13 crRNA (specific for each gene, see Table 1)

- Laminar flow hood (BSC Class II)
- Disposable, powder-free gloves
- Vortex mixer (VWR, 10153-838)
- Sterile, nuclease-free microcentrifuge tubes, 0.5-ml (Olympus Plastics, 24-272S or equivalent)
- Sterile, nuclease-free microcentrifuge tubes, 1.7-ml (Olympus Plastics, 24-282S or equivalent)
- Sterile, nuclease-free microcentrifuge tubes, 2.0-ml (Olympus Plastics, 24-273S or equivalent)
- 15-ml conical tubes (Olympus Plastics, 28-103 or equivalent)

Table 1 Primer, crRNA, *in vitro* Transcription Templates, and Cas13 Reporter Sequences^a

PCR primers	Sequence 5' to 3'
N1 fwd	<i>gaaat</i> TAATACGACTCACTATA <i>Agggcg</i> accccaaatcagcgaat
N1 rev	tctggttactgccagttgaatctg
N2 fwd	<i>gaaat</i> TAATACGACTCACTATA <i>Agggctt</i> acaacattggccgcaaa
N2 rev	gcgcgacattccgaagaa
RNaseP fwd	<i>gaaat</i> TAATACGACTCACTATA <i>Agggagatt</i> tgacctgcgagcg
RNaseP Rev	gtgagcggctgtctccaaa
crRNAs	Sequence 5' to 3'
N1 gRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACaggguccaccaacguaaugcggggugc
N2 gRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACgcugaagcgcugggggcaauugugcaa
RNase P gRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACguccgcgcagaccuucaggucagaacc
Reporter	Sequence 5' to 3'
CREST	6-FAM (Fluorescein) – (U) ₇ – Iowa Black FQ
Fluorescent	
Cleavage reporter	
Positive control	
synTarget N1	<i>gaaat</i> TAATACGACTCACTATA <i>Agggg</i> accccaaatcagcgaatgcaccccgcat tacgtttggtggaccctcagattcaactggcagtaaccaga
synTarget N2	<i>gaaat</i> TAATACGACTCACTATA <i>Agggtt</i> acaacattggccgcaaatgcacaatttgc ccccagcgttcagcgttctcgaatgtcgcgc

^aFor PCR primers, the minimal T7 RNA polymerase promoter is indicated in uppercase. Flanking sequences to increase transcription rates are indicated in italic font. For crRNAs, the shared Cas13 crRNA is denoted in uppercase. For the positive control, the minimal T7 RNA polymerase promoter is indicated in uppercase.

50-ml conical tubes (Olympus Plastics, 28-106 or equivalent)
 Hard-Shell® 96-well PCR Plates (BioRad, HSP9601)
 Microseal™ ‘B’ adhesive seal (BioRad, MSB1001)
 SimpliAmp thermocycler (Applied Biosystems, A24811 or equivalent 96-well thermocycler)
Optional: heat block/water bath
 Centrifuge with adapter for 96-well plates
 Single and multichannel adjustable pipettes (1 to 1000 µl)
 MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, ThermoFisher, 4309849)
 Quantstudio™ 5, BioRad CFX96, or equivalent real-time PCR detection system

NOTE: Control materials to be used with CREST:

A “no template” (negative or NTC) control is required to monitor cross-contamination during sample handling or test setup. All PCR and LwaCas13a reactions must include a “no template” control prepared using nuclease-free water instead of input RNA.

A positive template control is required to monitor the integrity of the test. All PCR and LwaCas13a reactions must include a positive control. The positive control can be prepared by *in vitro* transcription using the MEGascript™ T7 Transcription Kit (Fisher AM1334) with the sequences synTarget N2 and synTarget N3 as templates (see Support Protocol 2). As an alternative, purified SARS-CoV-2 genomic RNA (ATCC VR-1991D), or RNA isolated from confirmed positive cases, can be used as positive controls.

Reverse transcription

Each sample will be tested in three reactions: one for each target in the downstream analysis (N1, N2, and RNase P).

Position	Target Gene											
	N1	N2	RNaseP	N1	N2	RNaseP	N1	N2	RNaseP	N1	N2	RNaseP
A	S1	S1	S1	S9	S9	S9	S17	S17	S17	NTC	NTC	NTC
B	S2	S2	S2	S10	S10	S10	S18	S18	S18	S25	S25	S25
C	S3	S3	S3	S11	S11	S11	S19	S19	S19	S26	S26	S26
D	S4	S4	S4	S12	S12	S12	S20	S20	S20	S27	S27	S27
E	S5	S5	S5	S13	S13	S13	S21	S21	S21	S28	S28	S28
F	S6	S6	S6	S14	S14	S14	S22	S22	S22	S29	S29	S29
G	S7	S7	S7	S15	S15	S15	S23	S23	S23	S30	S30	S30
H	S8	S8	S8	S16	S16	S16	S24	S24	S24	POSITIVE	POSITIVE	POSITIVE

Figure 2 Loading map for Reverse Transcription. NTC, No Template Control.

1. Wipe the pre-amplification workstation surfaces and pipettes with RNase surface decontamination solution.
2. Prepare a working stock of gene-specific primers by mixing the forward and reverse primers at a final concentration of 5 μ M. See Table 1 for oligonucleotide sequences.

Primer mixes can be stored at -20°C for future use.

3. Prepare the RT master mix by adding reagents to a 0.2- or 0.5-ml tube in the order listed below (volumes for $N = 1$):

Prepare a separate RT master mix for each target to be detected (N1, N2, or RNaseP).

Calculate the number of reactions (N) to assemble and multiply by 1.2 to account for 20% pipetting error. Thaw enough reagents for $1.2 \times N$ reactions on ice. Include in the calculations one positive and one negative control sample. Keep all reagents and reaction products on ice unless otherwise stated.

- 1 μ l of gene-specific primer mix (5 μ M each primer)
- 2 μ l of 5 \times Reaction Buffer for RT
- 1 μ l of dNTPs (10 mM each dNTP)
- 0.5 μ l of 200 U/ μ l RevertAid Reverse Transcriptase (200 U/ μ l)
- 0.5 μ l of 40 U/ μ l Murine RNase Inhibitor.

4. Transfer 5 μ l of each master mix, N1, N2, and RNase P, to each well of a 96-well PCR plate.
5. Add 5 μ l of template RNA to each well. See Figure 2 for the suggested plate loading map.
6. Seal the plate with adhesive film.
7. Pulse-spin the plate to collect the contents at the bottom of the wells.
8. Incubate at 42°C for 30 min in a 96-well thermocycler.

Alternatively, incubate the reactions in a 42°C water or dry bath.

PCR amplification

9. Prepare the PCR master mix by adding reagents in the order listed below:

Calculate the number of reactions (N) to assemble and multiply by 1.2 to account for 20% pipetting error.

Make sure to include the positive and negative controls in the calculation. Thaw enough reagents for $1.2 \times N$ reactions on ice. Keep all reagents and reaction products on ice unless otherwise stated.

- 17 μ l of nuclease-free water
- 5 μ l of Taq PCR 5 \times Master Mix
- 1 μ l of gene-specific primer mix (5 μ M each primer).

Table 2 PCR Amplification Thermocycling Program

Temperature	Time	Number of cycles	Step
95°C	2 min	1 cycle	Initial denaturing
95°C	15 s	20 cycles	Denature
60°C	15 s		Anneal primers
72°C	15 s		Extend
72°C	5 min	1 cycle	Final extension

- Transfer 23 μl to each well of a 96-well plate following the loading map in Figure 2.
- Carefully transfer 2 μl from the RT reaction (see step 8) to the well containing the PCR master mix using a multichannel pipette. Follow the same gene/sample order as seen in Figure 2.

Match the target gene from the RT to the PCR. For example, the RT product of sample 1/N1, should be amplified by PCR with the N1 primers.

- Pulse-spin the plate to collect the contents at the bottom of the wells.
- Place the plate in the thermocycler and amplify the DNA using the thermocycling program shown in Table 2. Upon completion of the PCR reaction, place the plate on ice.
- Take precautions to avoid amplicon cross-contamination.

IMPORTANT NOTE: *Never open the plate containing the PCR products in the pre-amplification work area! Open the plate only in a designated post-amplification workstation.*

Target detection with Cas13

- Prepare a Cas13a detection master mix as follows.
Calculate the number of reactions (N) to assemble, and multiply by 1.2 to account for 20% pipetting error. Make sure to include the positive and negative controls in the calculation. Thaw enough reagents for $1.2 \times N$ reactions on ice. Keep all reagents and reaction products on ice unless otherwise stated.
Volumes in the list below are for 4 μl of master mix to be used in 384-well format. If working in 96-well format, double the volume of reagents to get 8 μl of the master mix.
 - 1.6 μl of nuclease-free water
 - 0.5 μl of 10 \times Cleavage Buffer (400 mM Tris·HCl, pH 7.5, 10 mM DTT)
 - 0.2 μl of rNTPs (25 mM each rNTP)
 - 0.25 μl of 40 U/ μl Murine RNase Inhibitor
 - 0.3 μl of 2 μM Cleavage Reporter (see Table 1)
 - 0.5 μl of 63 $\mu\text{g}/\text{ml}$ LwaCas13a
 - 0.15 μl of 50 U/ μl T7 RNA polymerase
 - 0.05 μl of Cas13 crRNA (specific for each gene, see Table 1).
- Aliquot 4 μl of the master mix into a 384-well plate, or 8 μl of the master mix into a 96-well plate.
- Carefully transfer 1 μl of the PCR reaction to the 384-well plate containing the Cas13a detection master mix, or 2 μl of the PCR reaction to the 96-well plate containing the Cas13a detection master mix, using a multichannel pipette. Follow the loading map in Figure 2.

Table 3 Valid Assay Criteria

	FL _{t30} /FL _{t0}	
	Negative control	Positive control
Invalid	>3	<5
Valid	<3	≥5

Be sure to transfer the PCR product for the gene of interest to the corresponding well with the gene-specific crRNA. For example, the PCR product amplified with the N1 primers will only be detected with the N1 crRNA. See Table 1 for all oligo sequences.

18. Pulse-spin the plate to collect contents at the bottom of the wells.
19. Take a fluorescence read for the FAM channel (excitation: 470 ± 15 nm; emission: 520 ± 15 nm) at time = 0 (FL_{t0} immediately after plate setup).
20. Incubate samples at 37°C in the thermocycler for 30 min, and take the final fluorescence read (FL_{t30}).

Data analysis and interpretation of results

21. Evaluate the validity of the assay (Table 3). The assay is considered invalid if:
 - a. The negative control signal at 30 min is at least three times higher than at time 0 (Neg control FL_{t30}/Neg control FL_{t0} ≥ 3). This result could indicate reagent contamination. Eliminate all samples, re-extract RNA, and repeat the reactions with fresh reagents.
 - b. The positive control signal at 30 min (FL_{t30}) is at least five times lower than at time 0 (Pos control FL_{t30}/Pos control FL_{t0} <5). This result indicates a failure of the reaction. Repeat the reaction.
 - c. In some cases, the FL_{t30} is lower than the FL_{t0} in the negative control or the samples. In that case, the assay is still valid.
 - d. If the assay controls are invalid, all reactions must be repeated and/or samples re-processed for RNA extraction.
22. Calculate the baseline fluorescence signal for the assay plate as follows:
 Neg control FL_{t30} – Neg control FL_{t0} = Baseline FL_{base}
If the baseline FL_{base} is a negative number, use the absolute value for all calculations.
23. Calculate the normalized signal for N1, N2, and RNaseP in each sample.
 Sample FL_{t30} – Sample FL_{t0} = Sample FL_{norm}
24. Determine if the sample is positive, negative, or inconclusive as follows:
 - a. Negative for SARS-CoV-2:
 All controls work as expected, the normalized RNaseP signal (FL_{norm}) is at least five times higher than the baseline signal (FL_{base}), and the N1 and N2 normalized signals (FL_{norm}) are five times lower than the baseline signal (FL_{base}).
 - b. Positive for SARS-CoV-2:
 - i. All controls work as expected, the normalized RNaseP signal (FL_{norm}) is at least five times higher than the baseline signal (FL_{base}), and the N1 and N2 normalized signals (FL_{norm}) are five times higher than the baseline signal (FL_{base}).
 - ii. All controls work as expected, the N1 and N2 normalized signal (FL_{norm}) is at least five times higher than the baseline FL_{base}, but the RNase P normalized signal (FL_{norm}) is not 5 times higher than the baseline signal (FL_{base}). Failure of RNaseP detection does not preclude the samples from carrying viral RNA.

This result likely indicates a low number of cells in the sample and low levels of RNaseP.

- c. Inconclusive:
- d. The controls perform as expected, but N1, N2, and RNase P are not detected. This result may indicate a problem with RNA extraction. Repeat the extraction and repeat the test.
- e. The controls perform as expected, the RNase P normalized signal (FL_{norm}) is five times or higher than the baseline signal (FL_{base}), and only N1 or N2 are detected. This result may indicate contamination or pipetting errors. Repeat the test. If the sample remains ambiguous, repeat the RNA extraction and the test.

ALTERNATE PROTOCOL

Cas13-BASED DETECTION OF SARS-CoV-2 GENETIC MATERIAL USING A FLUORESCENCE VIEWER

This protocol is an alternative to the Basic Protocol for endpoint detection of CREST reactions on the affordable P51™ Molecular Fluorescence Viewer from miniPCR®. This Alternate Protocol allows running CREST using an inexpensive thermocycler like the miniPCR® mini8 or mini16 and a compact handheld fluorescence viewer. Using this protocol, CREST can be performed at low cost, with minimal infrastructure, maintaining high sensitivity. Critical differences between this method and the one described in Basic Protocol are the use of the miniPCR thermocycler, detection using the P51 viewer miniPCR®, and the use of 8-well strip tubes instead of 96-well plates. This protocol is suggested for small numbers of samples, and/or field deployment.

Additional Materials (also see Basic Protocol)

- Cas13a storage buffer: 50 mM Tris·HCl, pH 7.5, 600 mM NaCl, 5% (v/v) glycerol, 2 mM DTT)
- 0.2 ml 8-Strip PCR Tubes (Olympus Plastics, 27-125U or equivalent)
- Mini centrifuge capable of spinning 8-tube PCR strips (Fisher, S67601B)
- miniPCR™ mini16 thermocycler (miniPCR, QP-1016-01)
- P51™ Molecular Fluorescence Viewer (miniPCR, KT-1100-01)
- Equipment for photography

Reverse transcription

Test each sample using three reactions: one for each target in the downstream analysis (N1, N2, and RNase P). Eight samples will require three 8-tube PCR strips.

1. Wipe the pre-amplification workstation surfaces and pipettes with RNase surface decontamination solution.
2. Prepare the RT master mix by adding reagents to a 0.2- or 0.5-ml tube in the order listed below:
You will have to prepare a separate RT master mix for each target to be detected (N1, N2, or RNaseP).
Calculate the number of reactions (N) to assemble and multiply by 1.2 to account for 20% pipetting error. Include a negative and positive control (see Support Protocol 2).
Thaw enough reagents for $1.2 \times N$ reactions on ice. Keep all reagents and reaction products on ice unless otherwise stated.

- 1 μ l of gene-specific (N1, N2 or RNaseP) primer mix (5 μ M each primer)
- 2 μ l of 5 \times Reaction Buffer for RT
- 1 μ l of dNTPs (10 mM each dNTP)
- 0.5 μ l of 200 U/ μ l RevertAid Reverse Transcriptase (200 U/ μ l)
- 0.5 μ l of 40 U/ μ l Murine RNase Inhibitor (40 U/ μ l).

3. Transfer 5 μ l of each master mix to an 8-tube PCR strip. Use one 8-tube PCR strip for each gene-specific master mix (one strip for N1, one strip for N2, one strip for RNase P).
4. Add 5 μ l of template RNA for each sample to the tubes containing N1, N2, and RNaseP master mix. Each sample is added to three separate tubes.
5. Close the caps on the strip tubes and pulse spin on a mini centrifuge to collect sample mixture at the bottom of the tubes.
6. Incubate at 42°C for 30 min in the miniPCR® thermocycler or similar equipment. Alternatively, incubate at 42°C water or dry bath.

PCR amplification

7. Prepare the PCR master mix by adding reagents in the order listed below:
Calculate the number of reactions (N) to assemble and multiply by 1.2 to account for 20% pipetting error. Include a positive and negative control. Thaw enough reagents for $1.2 \times N$ reactions, on ice. Keep all reagents and reaction products on ice unless otherwise stated.
 - 17 μ l of nuclease-free water
 - 5 μ l of Taq PCR 5 \times Master Mix
 - 1 μ l of gene-specific primer mix (5 μ M each primer).
8. Transfer 23 μ l of master mix to each tube of a new 8-tube PCR strip. Run each gene-specific mix in a separate 8-tube PCR strip (one strip for N1, one for N2, and one for RNase P).
9. Carefully transfer 2 μ l from the RT reaction to the tube containing the PCR master mix.
Match the gene-specific product with the correct PCR primers. For example, the RT product of sample 1/N1 should be amplified by PCR with the N1 primers. A multichannel pipette may be used to set up multiple reactions simultaneously.
10. Pulse-spin the tube strips to collect the contents at the bottom of the tubes.
11. Place the tube strips in a miniPCR® thermocycler or similar, and amplify the DNA using the thermocycling program in Table 2. Upon completion of the PCR reaction, place the tubes on ice.
12. Take precautions to avoid amplicon cross-contamination.

IMPORTANT NOTE: Never open the tube strips containing the PCR products in the pre-amplification work area! Only open the tube strips in a designated post-amplification workstation.

Target detection with Cas13

13. Prepare a Cas13a detection master mix as follows.
Calculate the number of reactions (N) to assemble and multiply by 1.2 to account for 20% pipetting error. Make sure to include the positive and negative controls in the calculation. Thaw enough reagents for $1.2 \times N$ reactions on ice. Keep all reagents and reaction products on ice unless otherwise stated.
Volumes in the list below are for 16 μ l of master mix for each sample.
 - 6.4 μ l of nuclease-free ultrapure water
 - 2 μ l of 10 \times Cleavage Buffer (400 mM Tris·HCl, pH 7.5, 10 mM DTT)
 - 0.8 μ l of rNTPs (25 mM each rNTP)
 - 1 μ l of 40 U/ μ l Murine RNase Inhibitor (40 U/ μ l)
 - 1.2 μ l of 2 μ M Cleavage Reporter (see Table 1)

- 2 μ l of 63 μ g/ml LwaCas13a
- 0.6 μ l of 50 U/ μ l T7 RNA polymerase
- 0.2 μ l of Cas13 crRNA (specific for each gene, see Table 1)
- 1.8 μ l of 100 mM MgCl₂.

14. Carefully transfer 4 μ l of the PCR reaction to the tube strips containing 16 μ l of the Cas13a detection master mix.
15. Pulse-spin the tube strips to collect the contents at the bottom of the tubes.
16. Inspect the samples in the p51 fluorescence viewer immediately after setup and confirm no fluorescence in any tube.
17. Incubate samples at 37°C for 30 min. Fluorescence is evaluated and the results are recorded by photography after 30 min. We recommend inspecting the samples and taking photographs in a dark room. Sample can be kept at 4°C for up to 1 hr before reading, and the signal is stable at 4°C for up to 24 hr.

Data analysis and interpretation of results

18. Evaluate if the assay is valid. The assay is considered invalid if:
 - a. Fluorescence is detected in the negative control for any marker (N1, N2, or RNase P). This result could indicate reagent contamination. Eliminate all samples, re-extract RNA, and repeat with fresh reagents.
 - b. The fluorescence signal is not detected in the positive control in any marker (N1, N2, or RNase P). This result indicates a failure of the reaction. Repeat the reaction.
19. Determine if the sample is positive, negative, or inconclusive as follows:
 - a. Negative for SARS-CoV-2:
 - i. All controls work as expected, the RNase P reaction shows detectable fluorescence, and there is no detectable fluorescence in N1 and N2 (lower or equal signal to the negative control).
 - b. Positive for SARS-CoV-2:
 - i. All controls work as expected, the RNase P shows detectable fluorescence, and there is detectable fluorescence in N1 and N2.
 - ii. All controls work as expected, there is detectable fluorescence in N1 and N2, and there is no fluorescent signal in RNase P. Failure of RNase P detection does not preclude the samples from carrying viral RNA. This result likely indicates a low number of cells in the sample and low levels of RNase P.
 - c. Inconclusive:
 - i. The controls perform as expected, but there is no fluorescence detected in N1, N2, and RNase P. This result may indicate a problem with the RNA extraction for that sample. Repeat the RNA extraction and repeat the test.

Samples that show signal in N1 but not N2 should be re-tested to confirm the results.

SUPPORT PROTOCOL 1

LwaCas13a PURIFICATION

This protocol describes the purification of the LwaCas13a enzyme required to detect target sequences as described in the Basic Protocol and Alternate Protocol. This protocol begins with the expression of recombinant, affinity-tagged form of LwaCas13a followed by purification by anion exchange chromatography.

Additional Materials (also see Basic Protocol)

- Rosetta 2(DE3)pLysS cells (Novagen, Millipore 71403)
- pC013: TwinStrep—SUMO—huLwCas13a expression plasmid (Addgene, 90097)

SOC medium (ThermoFisher, 15544034 or equivalent)
LB plates with 100 µg/ml ampicillin (see Current Protocols article: Elbing & Brent, 2018)
Terrific broth (TB; VWR, J869-500G or equivalent) with 100 µg/ml ampicillin (TB-Amp)
IPTG (Invitrogen, 15529019 or equivalent)
Lysis buffer: 20 mM Tris·HCl, pH 8.0, 500 mM NaCl, 1 mM DTT, plus 1 cOmplete™ mini protease inhibitor cocktail tablet per 10 ml of lysis buffer (Roche, 11836153001)
Strep-Tactin superflow agarose (Millipore, 71592)
SUMO protease (ThermoFisher 12588018)
NP-40
Buffer A: 20 mM Tris·HCl, pH 7.5 containing 5% (v/v) glycerol and 1 mM DTT
Buffer B: 20 mM Tris HCl, pH 7.5, 2 M NaCl, 5% (v/v) glycerol, 1 mM DTT
SEC buffer: 50 mM Tris·HCl, pH 7.5 containing 600 mM NaCl and 2 mM DTT
LwaCas13a storage buffer: 50 mM Tris·HCl, pH 7.5, 600 mM NaCl, 5% (v/v) glycerol, 2 mM DTT
Liquid nitrogen

37°C shaking incubator

Centrifuge

UV-Vis spectrophotometer

Probe-type sonicator

Pierce Centrifuge Column (ThermoFisher, 89898)

Rotator

FPLC System

Cation-exchange column (HiTrap SP HP, Cytiva, 17115201)

Amicon centrifugal filters (EMD Millipore, UFC905008)

Size exclusion column (Superdex200 10/300 GL, Cytiva, 28990944)

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2006)

1. Transform Rosetta 2(DE3)pLysS cells with the expression vector Twinstrep-SUMO-huLwCas13a.
 - a. Remove competent cells from the freezer and allow the cells to thaw on ice for 2-5 min.
 - b. Once cells are thawed, mix gently by flicking the tube one or two times.
 - c. Mix 50 µl of the cells with 1 µl of the TwinStrep–SUMO–huLwCas13a (100 ng/µl), and mix gently by flicking the tube. Never vortex competent cells. Incubate on ice for 5 min.
 - d. Incubate tubes for 30 s at 42°C. Never shake the tubes during incubation.
 - e. Immediately place the tubes on ice for at least 2 min. Keep the tubes on ice until SOC medium is added.
 - f. Add 250 µl of room temperature SOC medium.
 - g. Plate 100 µl of the cells in pre-warmed LB plates with 100 µg/ml ampicillin. Incubate overnight at 37°C.

You should expect hundreds of colonies. If you get no colonies, check the batch of antibiotic used and repeat the transformation. If no colonies appear, confirm the competence of the host cells.
2. Inoculate 25 ml of TB containing 100 µg/ml ampicillin (TB-Amp) with a single colony from the LB-Amp plate. Incubate overnight at 37°C, shaking at 300 rpm.

3. The following morning, incubate 1 L of TB-Amp with 10 ml of the starter culture. Incubate at 37°C/300 rpm until an OD₆₀₀ of 0.6 is achieved.
Monitor the OD of the culture at regular intervals to achieve the desired OD.
4. Once OD₆₀₀ has reached 0.6, cool incubator to 21°C.
5. Treat the cells with 500 μM IPTG overnight with shaking at 21°C.
6. Collect cells by centrifugation at 3000 × g for 15 min at 4°C. Resuspend the cell pellet (4× w/v, e.g., 40 ml for 10 g pellet) in ice-cold lysis buffer.
7. Sonicate the lysate, on ice, for 5 min, with an amplitude setting of 50% for 30 s ON and 30 s OFF. Collect 100 μl of lysate for SDS-PAGE analysis.
8. Clear the lysate by centrifugation for 30 min at 12,000 × g, 4°C.
9. Collect the supernatant (soluble fraction). Take a 100-μl aliquot of the clarified supernatant for SDS-PAGE analysis. Take an aliquot of the pellet and resuspend in 100 μl of the lysis buffer for SDS-PAGE analysis.
10. Add 5 ml of Strep-Tactin agarose to the soluble fraction and incubate for 2 hr at 4°C.
11. Secure a disposable centrifuge column and add the resin-sample solution. Collect the flowthrough and take a 100-μl aliquot for SDS-PAGE analysis.
12. Wash the resin with 25 ml of cold lysis buffer (without protease inhibitors). Take a small sample of the resin for SDS-PAGE analysis.
13. For resin cleavage of the SUMO tag, add 50 U of SUMO protease (1 U/μl in 15 ml of lysis buffer + 0.15% NP-40 with no protease inhibitors) to the column, cap the column, and incubate at 4°C overnight with gentle rotation.
14. The next day, drain the column to elute the LwaCas13a. Wash the column with an additional 15 ml of lysis buffer (without NP-40 or protease inhibitors) to ensure full collection. Collect 100-μl fraction for SDS-PAGE analysis.
15. Analyze all collected fractions by SDS-PAGE (see Current Protocols article: Gallagher, 2006) to ensure proper binding and elution of LwaCas13a from the Strep-Tactin column before proceeding.
16. Dilute the eluate two-fold with Buffer A to reduce NaCl concentration to 250 mM.
17. Load the purified LwaCas13 onto a cation-exchange column equilibrated with 5 column volumes of a mixture of Buffer A and 12.5% Buffer B in an FPLC system.
18. Wash the column with 5 column volumes of the Buffer A/12.5% Buffer B.
19. Gradient-elute LwaCas13a by ramping from 12.5% Buffer B to 100% Buffer B. LwaCas13a elutes at ~25% Buffer B. Use 10 column volumes at 5 ml/min.
20. Collect samples from peak fractions for SDS-PAGE analysis.
21. Pool the fractions containing LwaCas13a, concentrate them with Amicon centrifugal filter, and load them onto a SEC200 column equilibrated in SEC buffer.
22. Collect samples from peak fractions for SDS-PAGE analysis.
23. Pool the fractions containing LwaCas13a, concentrate with Amicon centrifugal filter, and buffer exchange into LwaCas13a storage buffer.
24. Determine LwaCas13a protein concentration by absorbance at 280 nm.

Typical yields are 7 mg/L.

25. Dilute purified LwaCas13a protein to 2 mg/ml in LwaCas13a storage buffer, aliquot, and flash freeze in liquid nitrogen. Store aliquots at -80°C.
26. Before use, dilute LwaCas13a to 63 µg/ml in LwaCas13a storage buffer.

IN VITRO TRANSCRIPTION OF SYNTHETIC TARGETS

This protocol describes the preparation of synthetic RNA targets corresponding to the N1 and N2 regions in the SARS-CoV-2 Nucleocapsid protein. These RNAs are to be used as positive controls. Synthetic template DNA oligonucleotides (N1 or N2), flanked by an upstream T7 RNA polymerase promoter, are annealed and PAGE purified prior to in vitro transcription.

Additional Materials (also see Basic Protocol)

Annealing buffer: 100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate
DNA ultramers for the N1 and N2 regions of the SARS-CoV-2 nucleocapsid gene (see Table 1 for oligonucleotide sequences)
Novex 4%-20% TBE PAGE gels (ThermoFisher, EC62252BOX)
10× TBE buffer: 1 M Tris·HCl, pH 8, 1 M boric acid, 20 mM EDTA
SYBR-Safe DNA Gel Stain (ThermoFisher, S33102 or equivalent)
TE buffer: 10 mM Tris·HCl, pH 8.5 containing 1 mM EDTA
100% isopropanol
3 M sodium acetate, pH 5.5 (Invitrogen, AM9740 or equivalent)
100% and 80% ethanol
Megascript T7 Transcription kit (ThermoFisher, AM1334)
Acid-phenol:chloroform, pH 4.5 (Invitrogen, 10095904)
Chloroform

Aluminum heat block
Clean razor blades
Transilluminator
1.5-ml microcentrifuge tubes
Shaker
UV-Vis spectrophotometer (Nanodrop, ThermoFisher)

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2006)

1. Prepare annealing buffer:
 - 125 µl of 8 M potassium acetate (final concentration, 100 mM)
 - 300 µl of 1 M HEPES-KOH pH 7.4 (final concentration, 30 mM)
 - 20 µl of 1 M magnesium acetate (final concentration, 2 mM)
 - 9.375 ml of nuclease-free water.
2. Dissolve the single-strand DNA ultramers in nuclease-free water at a concentration of 10 µg/µl.
3. Anneal the ultramers by mixing:
 - 25 µl of the 10 µg/µl fwd ultramer
 - 25 µl of the 10 µg/µl rev ultramer
 - 50 µl of the 2× annealing buffer.
4. Incubate at 95 °C for 5 min in an aluminum heat block.

5. Remove the block from the heating element and place it on the bench top to allow the annealing reactions to cool slowly to room temperature.
6. Separate the annealed ultramers from contaminants on a 4%-20% gradient TBE PAGE gel. Run the gel at 150 V.
7. Stain the gels with SYBR-Safe, diluted 1:10,000 in 1× TBE buffer, for 10 min.
8. Excise the annealed ultramers from the gel using a clean razor blade while visualizing in a UV transilluminator.
9. Place the gel fragments in 1.5-ml tubes, add 500 μl TE buffer, pH 8.5, and shake overnight at 700 rpm, 16 °C.
10. Transfer the TE buffer containing the eluted double-stranded DNA templates to a fresh tube.
11. Precipitate the template DNA with one volume of ice-cold isopropanol and one-ninth volume of 3 M sodium acetate, pH 5.5. Incubate at -80 °C for 30 min, and collect the DNA by centrifugation for 30 min at 20,000 × g, 4 °C, for 30 min.
12. Wash the DNA pellet with 1 ml ice-cold 80% ethanol and air-dry for 10 min. Resuspend the template DNA in 10 μl of nuclease-free water. Evaluate the concentration and purity using a UV spectrophotometer.
13. Transcribe the DNA templates using the Megascript T7 Transcription kit using 500 ng of PAGE-purified dsDNA as input for each reaction; all other reagents are added according to the manufacturer's protocol. Allow transcription to proceed overnight at 37 °C.
14. After transcription, remove the DNA templates by incubating the reaction mixture with 2 U of DNase (as detailed in the Megascript T7 Transcription kit) at 37 °C for 15 min. Add ammonium acetate (included in the Megascript T7 kit) to inactivate the DNases according to the manufacturer's protocol.
15. Purify the synthetic RNA targets by two sequential phenol extractions.
 - a. Add one volume of phenol:chloroform, pH 4.5, to the RNA.
 - b. Mix by vortexing and centrifuge 15 min at 12,500 × g, 4 °C.
 - c. Collect the aqueous phase and mix with one volume of chloroform.
 - d. Mix by vortex and centrifuge 15 min at 12,500 × g, 4 °C.
 - e. Collect the aqueous phase and repeat the extraction.
16. After the second extraction, precipitate the transcripts from the RNA-containing aqueous phase with one volume of ice-cold isopropanol and one ninth volume of 3 M sodium acetate, pH 5.5.
17. Incubate at -80 °C for 20 min and collect purified RNAs by centrifugation 20 min at 16,000 × g, 4 °C.
18. Wash the RNA pellet with 1 ml of ice-cold 80% ethanol and air dry for 10 min.
19. Resuspend the RNA in 10 μl of nuclease-free water.
20. Quantify the RNA and evaluate the quality using a UV spectrophotometer.

COMMENTARY

Background Information

CRISPR (clustered regularly interspaced short palindromic repeats) editing systems have gained prominence due to their vast po-

tential for use in multiple applications, including genome editing, molecule visualization, and gene activation or silencing, among others (Martin et al., 2012; Shivram, Cress,

Knott, & Doudna, 2021). Cas13 is a CRISPR-associated protein (Cas) RNA-guided-RNA nuclease. *In vitro* activation of Cas13 following binding to the target RNA leads to cleavage of the intended target, as well as to non-specific degradation of RNAs in the vicinity of the Cas13/RNA complex (Abudayyeh et al., 2017; Gootenberg et al., 2017). This “collateral activity” is the foundation of CREST. In CREST, Cas13 is guided by specific small CRISPR RNAs (crRNAs) to two regions of the SARS-CoV-2 nucleocapsid gene, or to the host RNase P transcript. Once Cas13 binds to its target (either N1, N2, or RNaseP), it will cleave nearby RNAs, including a fluorescein- and quencher-conjugated poly(U) RNA cleavage reporter. Reporter cleavage separates the quencher from the fluorophore, allowing for fluorescent signal detection. We increase the specificity and sensitivity of detection by using gene-specific primers for the RT and by including a short PCR amplification step.

The COVID-19 pandemic has highlighted the lack of scalable clinical diagnostic options available in locations with low levels of clinical infrastructure. These issues have been magnified by the limited availability and high cost of the reagents needed to monitor the prevalence of SARS-CoV-2. CREST addresses these issues by lowering the barrier to COVID-19 diagnostics, providing an assay that has high reagent accessibility and equipment availability and a low up-front and per-sample cost. CREST has been shown to be as sensitive as the CDC’s recommended TaqMan RT-qPCR assay (CDC, 2020; Rauch et al., 2021).

Critical Parameters and Troubleshooting

CREST’s sensitivity makes it particularly susceptible to contamination. To minimize the risk of contamination, this protocol should be tested in a facility following a unidirectional workflow. Specifically, it is essential to have separate work areas for pre-amplification and post-amplification work. Items from the post-amplification work area should be considered contaminated and never moved to the pre-amplification area. Reactions that have undergone amplification should never be opened anywhere other than in the post-amplification work area. Technicians should never take PPE, including gloves, lab coats, and goggles, from the post-amplification area to the pre-amplification area. In both the pre- and post-amplification areas, separate stations should be set up for master mix preparation and sam-

ple addition. Keeping these stations separated decreases the likelihood of contamination of the master mix for an entire set of samples. Positive and negative controls should be run with every sample.

Understanding Results

The analytical sensitivity of CREST for SARS-CoV-2 detection was determined in Limit of Detection (LoD) studies using the two qPCR instruments employed in the Basic Protocol (BioRad CFX96 and QuantStudio 5) and the blue LED fluorescence visualizer used in the Alternate Protocol (p51 Viewer, miniPCR). To determine the LoD for CREST using the real-time PCR instruments QuantStudio 5 and BioRad CFX96, we prepared samples in triplicate by spiking dilutions of heat-inactivated SARS-CoV-2 (ATCC VR-1986HK) into a negative nasopharyngeal/oropharyngeal sample matrix. We extracted the RNA using the QIAGEN QIAmp viral RNA mini kit, and CREST was performed as described in Basic Protocol. Samples were considered positive when the normalized fluorescence signal was at least 5 times higher than the negative control. Detection of RNaseP was used as an internal control.

The LoD for SARS-CoV-2 by CREST using the CFX96 was 200 viral genome copies/μl for N1 and 200 viral genome copies/μl for N2 (Fig. 3). When using the QuantStudio 5, the LoD for SARS-CoV-2 by CREST was 100 viral genome copies/μl for N1 and N2 (Fig. 3).

To determine the LoD of CREST using the p51 Fluorescence Viewer, we prepared samples in triplicate by spiking dilutions of heat-inactivated SARS-CoV-2 (ATCC VR-1986HK) into a negative nasopharyngeal/oropharyngeal sample matrix. We extracted the RNA using the QIAGEN QIAmp viral RNA mini kit, and CREST was performed as described in the Alternate Protocol. The LoD was determined as the lowest concentration at which 95% of the samples are positive. The estimated LoD for CREST using the p51 fluorescence viewer is 50 cp/μl for N1 and 200 copies/μl for N2 (Fig. 4).

To confirm the specificity of CREST detection of SARS-CoV-2, we spiked SARS-CoV-2 negative nasopharyngeal swab matrix with purified genomic RNA at 10⁵ copies/μl for common respiratory viral pathogens, or 10⁶ cells/ml of heat-inactivated bacterial pathogens commonly found in the respiratory tract. Samples were extracted and processed for CREST as described in the Basic Protocol or Alternate Protocol, and

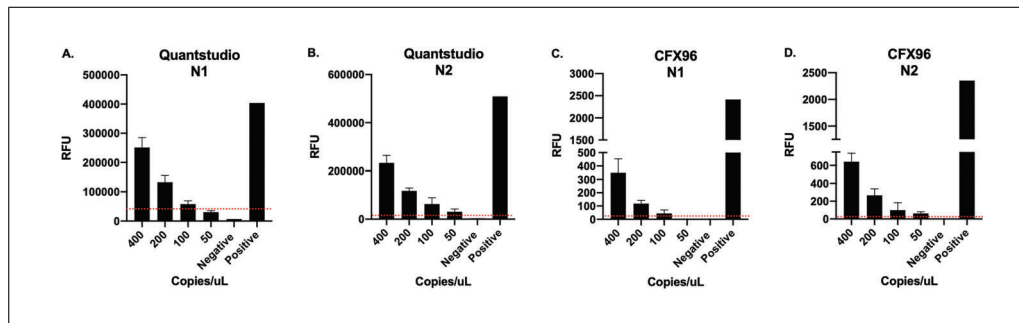


Figure 3 Estimation of LoD in the QuantStudio5 or the CFX96 real-time PCR instrument. Heat-inactivated SARS-CoV-2 was spiked at the indicated concentration in pooled negative NP/OP matrix ($N = 3$). Samples were extracted and CREST was performed as described to detect the N1 (A, C) or N2 (B, D) regions of the nucleocapsid (N) gene in the Quantstudio (A, B) or the CFX96 (C, D) real-time PCR instruments. The dotted line indicates $5\times$ the fluorescent signal from the negative controls (threshold of detection).

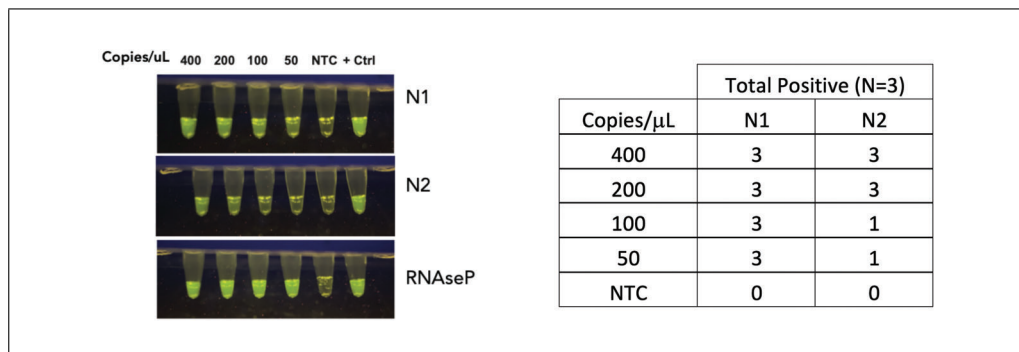


Figure 4 Estimation of LoD in the p51 fluorescence viewer. Heat-inactivated SARS-CoV-2 was spiked at the indicated concentration in pooled negative NP/OP matrix. Samples were extracted and CREST was performed as described. The image in (A) is representative of three replicates. (B) Summary of N1 or N2 detection using the p51 fluorescence viewer. Detection of RNaseP was used as an internal control. NTC; negative control.

Table 4 Cross-Reactivity Evaluation of CREST^a

Microorganism	QuantStudio5		CFX96		p51 viewer	
	N1	N2	N1	N2	N1	N2
SARS coronavirus	0/3	0/3	0/3	1/3	0/3	0/3
MERS coronavirus	0/3	0/3	0/3	0/3	0/3	0/3
Human Influenza A virus (H1N1)	0/3	0/3	0/3	0/3	0/3	0/3
<i>Streptococcus aureus</i>	0/3	0/3	0/3	0/3	0/3	0/3
<i>Streptococcus pneumoniae</i>	0/3	0/3	0/3	0/3	0/3	0/3
<i>Bordetella pertussis</i>	0/3	0/3	0/3	0/3	0/3	0/3
<i>Klebsiella pneumoniae</i>	0/3	0/3	0/3	0/3	0/3	0/3
<i>Pseudomonas aeruginosa</i>	0/3	0/3	0/3	0/3	0/3	0/3

^a Pooled negative NP/OP matrices were spiked with purified viral genomes (10^5 copies/ μ l) or heat-inactivated bacteria cells (10^6 cells/ml) in triplicate. Samples were extracted and CREST was performed as described using the N1 or N2 compatible primers and gRNAs. The table indicates the number of samples that had fluorescence over background.

fluorescence was detected using the QuantStudio 5, CFX96, or p51 viewer instruments. Using CREST, we were not able to detect common respiratory pathogens (Table 4). Only one sample of three replicates spiked with SARS-

CoV genome had a low CREST signal (close to the threshold of detection) with the N2 primer and gRNA, in the CFX96 instrument. These results indicate that CREST is highly specific for SARS-CoV-2 detection. For more

information, see the sections of the Basic Protocol and the Alternate Protocol entitled “Data analysis and interpretation of results.”

Time Consideration

Basic Protocol and Alternate Protocol will each take approximately 4 hr to perform. If many samples are being tested, then the required time may increase. Support Protocols 1 and 2 contain an overnight incubation step and require multiple days to complete. Support Protocol 1 will require approximately 5–6 days to complete, and Support Protocol 2 will require approximately 3 days to complete.

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Author Contributions

Zach Aralis: conceptualization, writing original draft, writing review and editing; **Jennifer N. Rauch:** conceptualization, data curation, formal analysis, methodology, writing original draft, writing review and editing; **Morgane Audouard:** data curation, investigation, methodology, writing review and editing; **Eric Valois:** conceptualization, investigation; **Ryan S. Lach:** data curation, investigation; **Sabrina Solley:** data curation, investigation; **Naomi J. Baxter:** data curation, investigation; **Kenneth S. Kosik:** conceptualization, funding acquisition, project administration, supervision, writing original draft, writing review and editing; **Maxwell Z. Wilson:** conceptualization, funding acquisition, investigation, methodology, project administration, supervision, writing original draft, writing review and editing; **Diego Acosta-Alvear:** conceptualization, funding acquisition, investigation, methodology, project administration, supervision, writing original draft, writing review and editing; **Carolina Arias:** conceptualization, data curation, formal analysis, methodology, project administration, supervi-

sion, validation, writing original draft, writing review and editing.

Conflict of Interest

Kosik, K., Wilson, M., Acosta-Alvear, D., and Arias, C. reported a patent for UC Case 2020-715 pending and being a coinventor in a provisional patent filed with the University of California, Santa Barbara (UCSB), Office of Technology and Industry Alliances for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection method known as CREST (Cas13-based, rugged, equitable, scalable testing) and a patent for PEARL, a method for nucleic acid isolation.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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