A protocol for detection of COVID-19 using CRISPR diagnostics

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*** As we continue to optimize this protocol, we will be uploading updated versions.

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*** Groups interested in testing this protocol using patient samples can request guidance by emailing sherlock@broadinstitute.org, and a limited number of starter samples of the Cas13 enzyme, crRNA, and lateral flow reporter are available on request

*** **IMPORTANT NOTE: <u>This protocol should not be used for clinical purposes.</u>** We do not have access to patient samples and as a result have not been able to validate this assay using real patient samples. We welcome researchers working with COVID-19 samples to contact us and we can provide a starter kit to test this system on. We hope this protocol will provide some reference points for researchers interested in further advancing this diagnostics system and also welcome researchers to contact us for assistance or guidance.

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Declaration of conflicts of interest: F.Z., O.O.A., and J.S.G. are inventors on patents related to Cas13, SHERLOCK, and CRISPR diagnostics, and are co-founders, scientific advisors, and hold equity interests in Sherlock Biosciences, Inc.

A. Overview

The recent outbreak of the novel coronavirus COVID-19 can be diagnosed using qPCR, but inadequate access to reagents and equipment has slowed disease detection. To help advance the diagnosis of COVID-19, we describe here a protocol for using the CRISPR-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) technique for the detection of COVID-19. Using synthetic COVID-19 virus RNA fragments, we have been able to consistently detect COVID-19 target sequences in a range between 20 and 200 aM (10-100 copies per microliter of input). The test can be carried out starting with RNA purified from patient samples, as is used for qRT-PCR assays, and can be read out using a dipstick in less than an hour, without requiring elaborate instrumentation.

The SHERLOCK COVID-19 detection protocol works in three steps and can be completed in 1 hour, starting from nucleic acid extraction as used for qRT-PCR tests.
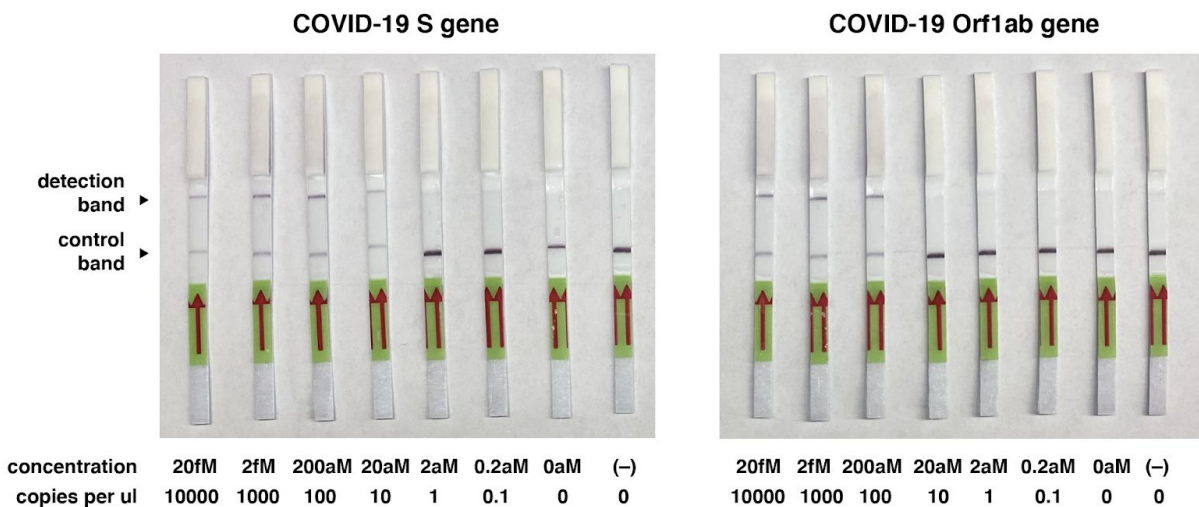
Step (1) – 25 min incubation – isothermal amplification of the extracted nucleic acid sample using a commercially available recombinase polymerase amplification (RPA) kit;
Step (2) – 30 min incubation – detection of pre-amplified viral RNA sequence using Cas13;
Step (3) – 2 min incubation – visual read out of the detection result by eye using a commercially-available paper dipstick.

The following sections detail the required reagents as well as procedure.

B. Assay Specifications

To check for the presence of COVID-19 RNA in nucleic acid extractions of specimens, two targets were chosen in the COVID-19 genome from the S gene and Orf1ab gene. RPA amplification primers and LwaCas13a CRISPR guide RNAs were designed for specific detection. In order to maximize the specificity of the assay, we selected guide sequences that minimized off-targets to related human respiratory virus genomes.

Using serial dilutions of synthetic COVID-19 S gene and Orf1ab gene RNA fragments, we were able to detect the presence of synthetic COVID-19 RNA sequence in a range between 10-100 copies per microliter. Below are example images of the lateral flow readout for different input concentrations:

	COVID-19 S gene								COVID-19 Orf1ab gene							
concentration	20fM	2fM	200aM	20aM	2aM	0.2aM	0aM	(−)	20fM	2fM	200aM	20aM	2aM	0.2aM	0aM	(−)
copies per ul	10000	1000	100	10	1	0.1	0	0	10000	1000	100	10	1	0.1	0	0

C. Materials and Reagents

Specimen and nucleic acid extraction:

The patient sample should be collected according to the appropriate biosafety procedures. Please reference the 2020 CDC COVID-19 test protocol for details on specimen collection and subsequent nucleic acid extraction. The input for this protocol, beginning with Step (1), can be the same extracted nucleic acid as used in qRT-PCR assays.

Reagents:

For Step (1) isothermal amplification:
- TwistAmp® Basic (TABAS03KIT), TwistDx Limited
- ProtoScript® II Reverse Transcriptase (M0368L), New England BioLabs
- RPA Amplification primer pair targeting S gene (S-RPA-Forward_v1: 5'-GAAATTAATACGACTCACTATAGGGAGGTTTCAAACTTTACTTGCTT TACATAGA-3'; S-RPA-Reverse_v1: 5'-TCCTAGGTTGAAGATAACCCACATAATAAG-3'), can be ordered from IDT
- RPA Amplification primer pair targeting Orf1ab (Orf1ab-RPA-Forward_v1: 5'-GAAATTAATACGACTCACTATAGGGCGAAGTTGTAGGAGACATTAT ACTTAAACC-3'; Orf1ab-RPA-Reverse_v1: 5'-TAGTAAGACTAGAATTGTCTACATAAGCAGC-3'), can be ordered from IDT

For Step (2) detection of virus RNA using Cas13:
- Cleavage buffer (400mM Tris pH 7.4 in ddH2O). Prepared using 1M Tris pH 7.4 stock solution from Sigma Aldrich (T2194-100ML)
- SUPERase•In™ RNase Inhibitor (AM2694), ThermoFisher Scientific
- NxGen® T7 RNA Polymerase (30223-1), Lucigen
- Ribonucleotide Solution Set (N0450S), New England BioLabs
- Magnesium chloride solution (63069-100ML), Sigma Aldrich
- Storage Buffer for diluting Cas13 protein stock (combine 2.5 mL of 1M Tris pH 7.4, 6 mL of 5M NaCl, 2.5 mL of glycerol, and 100 µL of 1M DTT, and 38.9 mL ddH2O)
- LwaCas13a protein purified according to Kellner et al., Nature Protocols 2019, stored as 4ul aliquots at 2mg/mL.
- LwaCas13a crRNA for detecting S gene (S-crRNA_v1: 5'-GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACGCAGCACC AGCUGUCCAACCUGAAGAAG-3'), can be ordered from Synthego
- LwaCas13a crRNA for detecting Orf1ab gene (Orf1ab-crRNA_v1: 5'-GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACCCAACCUCU UCUGUAAUUUUUAAACUAU-3'), can be ordered from Synthego
- Reporter RNA for lateral flow read out (Lateral-Flow-Reporter: 5'-/56-FAM/mArArUrGrGrCmAmArArUrGrGrCmA/3Bio/-3'), can be ordered from IDT

For Step (3) reading out using lateral flow dipstick:
- HybriDetect Dipstick (MGHD 1), Milenia Biotec GmbH

Positive control sequences (can be produced using T7 transcription from synthesized DNA oligos):
- S gene fragment:
  5'-UAACAUCACUAGGUUUCAAACUUUACUUGCUUUACAUAGAAGUU AUUUGACUCCUGGUGAUUCUUCUUCAGGUUGGACAGCUGGUGCUGC AGCUUAUUAUGUGGGUUAUCUUCAACCUAGGACUUUUCUAUUAAAA UAUAAUGAAAAUGGAACCAUUACAGAUGCUGUAGACUGUGC-3'
- Orf1ab gene fragment:
  5'-UGAGUGUAAUGUGAAAACUACCGAAGUUGUAGGAGACAUUAUAC UUAAACCAGCAAAUAAUAGUUUAAAAAUUACAGAAGAGGUUGGCCA CACAGAUCUAAUGGCUGCUUAUGUAGACAAUUCUAGUCUUACUAUU AAGAAACCUAAUGAAUUAUCUAG-3'

Equipment:
- 37C water bath
- 42C water bath
- Microcentrifuge for spinning down 1.5mL test tubes

D. Experimental Protocol

***IMPORTANT NOTE: To prevent sample contamination from confounding detection result, two different work areas should be used for performing Steps (1) and (2). Step (1) should be performed in a pre-amplification area and is especially sensitive to contamination. Amplified samples should not be opened in the work area for Step (1). A separate area for post-amplification reactions should be used for performing Step (2) of the protocol. After incubation, reactions from Step (1) should only be opened in the post-amplification area. Additionally only perform reactions from Step (3) in the post-amplification area.

***IMPORTANT NOTE: All reactions should be set up on ice prior to incubation at the designated temperature.

Step (1) – Isothermal Amplification, to be performed in the pre-amplification work area:

For testing each sample, set up two RPA reactions, for detection of the S gene target and Orf1ab target respectively. In addition, positive controls for S gene and Orf1ab can be set up using a synthetic virus fragment. A negative control without test samples added should also be set up.

Resuspend each lyophilized RPA pellet using 29.5ul of the Rehydration Buffer supplied in the RPA kit.

For the S gene target, each reaction can be set up as follows:

1. Resuspended RPA solution	5.9 ul
2. S-RPA-Forward_v1 (10 uM)	0.5 ul
3. S-RPA-Reverse_v1 (10 uM)	0.5 ul
4. ProtoScript RT (100,000U/mL)	0.2 ul
5. ddH2O	1.4 ul

6. Sample	1 ul
7. MgAc (supplied in RPA kit)	0.5 ul
Total	**10 ul**

For the Orf1ab gene target, each reaction can be set up as follows:

1. Resuspended RPA solution	5.9 ul
2. Orf1ab-RPA-Forward_v1 (10 uM)	0.5 ul
3. Orf1ab-RPA-Reverse_v1 (10 uM)	0.5 ul
4. ProtoScript RT (100,000U/mL)	0.2 ul
5. ddH2O	1.4 ul
6. Sample	1 ul
7. MgAc (supplied in RPA kit)	0.5 ul
Total	**10 ul**

Mix thoroughly and incubate each reaction at 42°C for 25 minutes in the pre-warmed water bath. After incubation, place the reaction back on ice immediately until ready to add to the reaction in Step (2).

Step (2) – Detection of viral RNA sequence using Cas13

Take one aliquot of the stock LwaCas13a protein (2 mg/mL, 4 ul), resuspend using 122.5 uL of Storage Buffer.

For each S gene RPA reaction, set up a Cas13 detection reaction as follows:

1. Cleavage Buffer (400mM Tris pH 7.4)	2 ul
2. ddH2O	9.6 ul
3. LwaCas13a protein (resuspended)	2 ul

4. S-crRNA_v1 (10 ng/ul)	1 ul
5. Lateral-Flow-Reporter (20 uM)	1 ul
6. SUPERase•In RNase Inhibitor	1 ul
7. Lucigen T7 Polymerase	0.6 ul
8. Ribonucleotide Solution	0.8 ul
9. MgCl2 (120mM)	1 ul
10. RPA reaction from Step (1)	1 ul
Total	**20 ul**

For each Orf1ab gene RPA reaction, set up a Cas13 detection reaction as follows:

1. Cleavage Buffer (400mM Tris pH 7.4)	2 ul
2. ddH2O	9.6 ul
3. LwaCas13a protein (resuspended)	2 ul
4. Orf1ab-crRNA_v1 (10 ng/ul)	1 ul
5. Lateral-Flow-Reporter (20 uM)	1 ul
6. SUPERase•In RNase Inhibitor	1 ul
7. Lucigen T7 Polymerase	0.6 ul
8. Ribonucleotide Solution	0.8 ul
9. MgCl2 (120mM)	1 ul
10. RPA reaction from Step (1)	1 ul
Total	**20 ul**

After all reactions are set up, vortex to mix thoroughly, spin down in a centrifuge, and incubate at 37°C for 30 minutes in a pre-warmed water bath. After incubation, place reaction tubes back on ice and proceed to Step (3) for lateral flow read out.

Step (3) – Visual readout of detection result via lateral flow strip

After Step (2), add 80 ul of HybriDetect Assay Buffer to each 20ul reaction and mix thoroughly. Place the diluted reaction in a tube rack at room temperature.

Place a HybriDetect Dipstick into each reaction tube and wait for the reaction to flow through the dipstick.

Positive control samples should show two lines and negative control samples should only show the bottom line.

For each test sample, check to see whether two lines appear for both S and Orf1ab genes, indicating a positive COVID-19 result.

E. Additional Information

A detailed general protocol for setting up SHERLOCK-based detection can be found in the following reference:

- *SHERLOCK: nucleic acid detection with CRISPR nucleases.* Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO, and Zhang F. *Nature Protocols*. 2019 Oct;14(10):2986-3012. doi: 10.1038/s41596-019-0210-2.