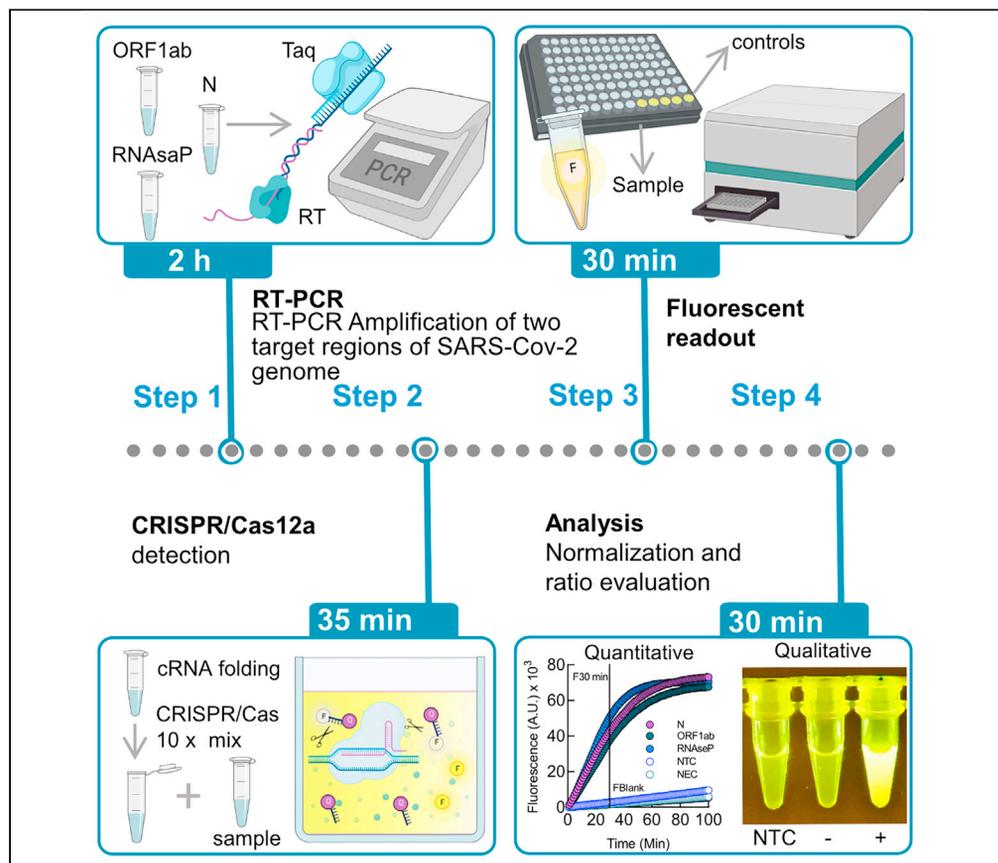


## Protocol

# UnCovid: A versatile, low-cost, and open-source protocol for SARS-CoV-2 RNA detection



Here, we describe a detailed step-by-step protocol to detect SARS-CoV-2 RNA using RT-PCR-mediated amplification and Crispr/Cas-based visualization. The optimized assay uses basic molecular biology equipment such as conventional thermocyclers and transilluminators for qualitative detection. Alternatively, a fluorescence plate reader can be used for quantitative measurements. The protocol detects two regions of the SARS-CoV-2 genome in addition to the human RNaseP sample control. Aiming to reach remote regions, this work was developed to use the portable molecular workstation from BentoLab.

Roberto Alcántara,  
Katherin Peñaranda,  
Gabriel  
Mendoza-Rojas, ...,  
Daniela Alvarez,  
Vanessa Adauí, Pohl  
Milón

pcmeralc@upc.edu.pe  
(R.A.)  
pmilon@upc.edu.pe  
(P.M.)

### Highlights

Molecular detection of SARS-CoV-2 using RT-PCR coupled to CRISPR/Cas technology

Compatibility for applying UnCovid as a Laboratory-developed test (LDT)

Protocol validated with clinical samples

Versatility for developing molecular detection of other RNA viruses

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## Protocol

## UnCovid: A versatile, low-cost, and open-source protocol for SARS-CoV-2 RNA detection

Roberto Alcántara,<sup>1,2,\*</sup> Katherin Peñaranda,<sup>1</sup> Gabriel Mendoza-Rojas,<sup>1</sup> Jose A. Nakamoto,<sup>1</sup> Eva Dueñas,<sup>1</sup> Daniela Alvarez,<sup>1</sup> Vanessa Aداui,<sup>1</sup> and Pohl Milón<sup>1,3,\*</sup>

<sup>1</sup>Centre for Research and Innovation, Health Sciences Faculty, Universidad Peruana de Ciencias Aplicadas (UPC), Lima 15023, Peru

<sup>2</sup>Technical contact

<sup>3</sup>Lead contact

\*Correspondence: [pcmeralc@upc.edu.pe](mailto:pcmeralc@upc.edu.pe) (R.A.), [pmilon@upc.edu.pe](mailto:pmilon@upc.edu.pe) (P.M.)  
<https://doi.org/10.1016/j.xpro.2021.100878>

## SUMMARY

Here, we describe a detailed step-by-step protocol to detect SARS-CoV-2 RNA using RT-PCR-mediated amplification and CRISPR/Cas-based visualization. The optimized assay uses basic molecular biology equipment such as conventional thermocyclers and transilluminators for qualitative detection. Alternatively, a fluorescence plate reader can be used for quantitative measurements. The protocol detects two regions of the SARS-CoV-2 genome in addition to the human RNaseP sample control. Aiming to reach remote regions, this work was developed to use the portable molecular workstation from BentoLab. For complete details on the use and execution of this protocol, please refer to Alcántara et al., 2021.

## BEFORE YOU BEGIN

The UnCovid protocol couples nucleic acid amplification using RT-PCR with detection of the amplified DNA mediated by CRISPR/Cas12a (Chen et al., 2018). Cas12a is a DNA nuclease that uses an adaptor RNA (CRISPR RNA) to identify the target DNA to be cleaved (Zetsche et al., 2015). Upon cleavage of the target double strand DNA (dsDNA), Cas12a activates a promiscuous off target activity that can cut single stranded DNA (ssDNA) (Jinek et al., 2012). This off target activity is used to cleave a dually labeled ssDNA. i.e., a ssDNA labeled with a donor fluorophore and a silent acceptor (quencher) results in measurable increase of fluorescence upon Cas12a-mediated cleavage. The Förster Resonance Energy Transfer (FRET) between the dyes is released due to a distance increase between the dyes. The UnCovid protocol is intended to be used with locally produced enzymes (M-MLV reverse transcriptase, Taq DNA polymerase, and LbCas12a) or their commercial counterparts of your preference. Albeit the crRNA can be chemically synthesized and purchased, *in vitro* transcription is most cost efficient. This segment of the protocol describes the production, purification, quantification, and storage of crRNA for CRISPR/Cas reaction. For details about enzyme production refer to (Mendoza-Rojas et al., 2021).

## General experimental considerations

1. This protocol is intended to be executed by experienced personnel. Users require to have a basic molecular biology training and experience, be accustomed with proper pipetting technique and trained for good clinical laboratory practice (GCLP).
2. Good clinical laboratory practices (GCLP) are recommended for use of this protocol. A 90 min online training in GCLP is available at the following link: <https://www.cdc.gov/labtraining/training-courses/good-lab-practices-molecular-genetics-testing.html>.



3. Correct use of basic equipment is necessary to avoid cross contamination. Avoid using the same gloves across all areas, amplicon contamination must be strongly contained (Davidi et al., 2021).
4. It is recommended to work with a unidirectional flow of samples considering “clean” (preparing reactions, sample manipulation) and “dirty” lab areas (where the thermal cyclers are located, and for post-processing steps (electrophoresis)). A Basic Molecular Biology Moduls for Laboratory Practice is available at the following link: <https://www.cdc.gov/labtraining/training-courses/basic-molecular-biology/laboratory-practice.html>

### crRNA purification

⌚ Timing: 4 h

#### 5. *In vitro* transcription reaction

*In vitro* transcription (IVT) requires a double-stranded DNA (dsDNA) template containing the T7 promoter sequence. Here, we used the following dsDNA templates to transcribe crRNAs, complementary to two SARS-CoV-2 genes and for the human RNaseP as sample quality control:

Gene	Sequence (5' – 3')
ORF1ab	TAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGATTTAGAGACGGTTGGGAAATTG
N	TAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGATCCCCAGCGCTTCAGCGTTC
RNaseP	TAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGATAATTACTTGGGTGTGACCCT

\*The T7 promoter sequence is highlighted in red.

**Note:** Use the *in vitro* transcription kit of your choice to prepare crRNA. Here, the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher) was used.

⚠ **CRITICAL:** Pay attention to the following general recommendations to avoid RNase contamination (Green and Sambrook, 2019): (1) Maintain a separate pipette set, pipette tips, and any other material required for RNA lab work. (2) Use gloves at all times. Change them frequently. (3) Preferably use RNase-free water (i.e., nuclease-free water aliquots) to prepare reactions or buffers. (4) Clean working surfaces, pipettes, and equipment with 20% bleach or alternative commercial solutions. To avoid accelerated deterioration of any plastics and metals, wipe down with 70% ethanol after using 20% bleach.

- a. Dissolve the dsDNA templates using nuclease-free water to 100  $\mu$ M concentration.
- b. Thaw all reagents and maintain the enzyme mix on ice.
- c. Prepare the 25 mM NTPs (ATP, GTP, CTP, and UTP) mix by adding an equal volume of each nucleotide (100 mM stock) in a single tube.
- d. Prepare the master mix for 40  $\mu$ L reactions as follows:

Reagent	Initial concentration	Final concentration	Volume ( $\mu$ L) per reaction	Volume ( $\mu$ L) for 3 reactions
5x Reaction buffer	5x	1x	8	24
NTP mix	25 mM	10 mM	16	48
Enzyme mix	-	-	4	12
Nuclease-free water <sup>a</sup>	-	-	11	33
Final volume			39	117

<sup>a</sup>Water volume can be adjusted depending on template volume

- e. Dispense 39  $\mu\text{L}$  of master mix in a 1.5 mL tube.
- f. Add 4  $\mu\text{g}$  of the respective dsDNA template to the reaction tube. Here, one microliter of a 100  $\mu\text{M}$  dsDNA stock (100 pmoles) was used.
- g. Incubate the reaction at 37°C for 3 h.

6. crRNA purification

**Note:** Use the RNA purification kit of your choice to purify the transcribed crRNA. Here, the Direct-zol RNA Miniprep purification Kit from Zymo Research was used.

- a. Add 3 volumes (i.e., 120  $\mu\text{L}$  for a 40  $\mu\text{L}$  reaction) of TRI-Reagent® (Zymo Research) or other similar product (TRIzol®, RNAzol®, TriPure™, or TriSure™) to each volume of the *in vitro* transcription reaction. Mix thoroughly and spin briefly.
- b. Add 1 volume (i.e., 160  $\mu\text{L}$ ) of 95%–100% cold ethanol. Mix thoroughly and spin briefly.
- c. Transfer the mixture to the spin column and centrifuge at 10000  $\times g$  for 30 s. Discard the flow-through (FT).
- d. Add 400  $\mu\text{L}$  of RNA wash buffer and centrifuge at 10000  $\times g$  for 30 s. Discard the FT.

**△ CRITICAL:** DNase I treatment is recommended for DNA template removal. Deoxyribonuclease I (DNase I) cuts both single-stranded and double-stranded DNA. Recombinant DNase I is typically used for selectively degrading DNA in the presence of RNA. One application of the DNase I treatment is removal of DNA template after *in vitro* transcription prior to downstream applications. Here, the DNase I and digestion buffer are provided with the Direct-zol RNA Miniprep kit (Zymo Research, Cat # E1010). The lyophilized enzyme can be reconstituted with 275  $\mu\text{L}$  of MilliQ water. DNase I digestion step is performed on the spin column. Prepare the working stock of DNase I by mixing 75  $\mu\text{L}$  of DNA digestion buffer (provided in the kit) with 5  $\mu\text{L}$  of DNase I (6 U/ $\mu\text{L}$  stock concentration, 0.38 U/ $\mu\text{L}$  final concentration) (Zymo Research, Cat # E1010). Alternatively, use DNase I in 1  $\times$  DNase I buffer (10 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, pH 7.6 25°C).

- e. Add 80  $\mu\text{L}$  of DNase I working stock to the column and incubate at 25°C for 15 min.
- f. Add 400  $\mu\text{L}$  of RNA pre-wash buffer and centrifuge at 10000  $\times g$  for 30 s. Discard the FT.
- g. Add 700  $\mu\text{L}$  of RNA wash buffer and centrifuge at 10000  $\times g$  for 30 s. Discard the FT.
- h. Transfer the column to a clean collection 1.5 mL tube
- i. Add 80  $\mu\text{L}$  of nuclease-free water. Incubate for 2–5 s, and centrifuge at 10000  $\times g$  for one minute. Collect the tube and place it on ice.
- j. Quantify the RNA by measuring the absorbance at 260 nm (A<sub>260</sub>) in a spectrophotometer. Here, the NanoDrop One microvolume UV-Vis spectrophotometer was used (Thermo Fisher Scientific). An A<sub>260</sub> nm absorbance of 1 is equivalent to 40  $\mu\text{g}/\text{mL}$  single-stranded RNA.
- k. Calculate the molar concentration using the corresponding molecular weight (ORF1ab = 13.5 kDa, N = 13 kDa, RNaseP = 13 kDa).
- l. Dilute your RNA stocks to 10–20  $\mu\text{M}$  using pre-cooled nuclease-free water, mix briefly and spin down.
- m. Dispense 20  $\mu\text{L}$  aliquots in 0.5–1.5 mL tubes and store at –80°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Nasopharyngeal swabs	Instituto de Investigación Nutricional, Lima, Peru	N/A
Quantitative PCR (qPCR) control RNA from heat-inactivated SARS-CoV-2, Isolate USA-WA1/2020	Centers for Disease Control and Prevention	BEI Resources NR-52347

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Potassium chloride	Supelco	Cat # 104936
Magnesium chloride hexahydrate	Supelco	Cat # 105833
Triton X-100	Sigma-Aldrich	Cat # 10789704001
Trehalose	Sigma-Aldrich	Cat # T9449-25G
Sodium chloride	Supelco	Cat # 106404
Glycerol 85%	Supelco	Cat # 104094
EDTA	Merck Millipore	Cat # 324503
Tris, Hydrochloride, Molecular Biology Grade	Merck Millipore	Cat # US1648317
Tris base ULTROL grade	Calbiochem	Cat # 643811
Dithiothreitol (DTT)	Thermo Fisher	Cat # R0682
dNTP Mix (10 mM each)	Thermo Fisher	Cat # R0194
SafeGreen	abm	Cat # G108-G
TriTrack DNA loading dye 6x	Thermo Fisher	Cat # R1161
Agarose powder	Cleaver Scientific	Cat # CSL-AG500
OmniPur Water DEPC treated, sterile, nuclease-free	Sigma-Aldrich	Cat # 9610-1L
DNaseI	PanReac Applichem	Cat # A3778
GeneRuler 100 bp DNA Ladder	Thermo Scientific	Cat # SM0242
Bovine Serum Albumin, lyophilized powder	Sigma-Aldrich	Cat # A9418-100G
<b>Critical commercial assays</b>		
TranscriptAid T7 High Yield Transcription Kit	Thermo Fisher	Cat # K0441
Direct-zol RNA Miniprep	Zymo Research	Cat # R2051
2x One-Step RT-PCR Master Mix	Norgen BioTek	Cat # 28113
QIAamp viral RNA Mini Kit	Qiagen	Cat # 52906
<b>Deposited data</b>		
Data S1 and S2	This paper	<a href="https://doi.org/10.17632/8m8z37v8xz.1">https://doi.org/10.17632/8m8z37v8xz.1</a>
<b>Oligonucleotides</b>		
dsDNA oligo: crRNA sequence N target SARS-CoV-2 TAATACGACTCACTATAGGTAATTTCTACTAAGTGT AGATCCCCAGCGCTTCAGCGTTC	<a href="#">Broughton et al.,2020</a>	N/A
dsDNA oligo: crRNA sequence ORF1ab target SARS-CoV-2 TAATACGACTCACTATAGGTAATTTCTACTA AGTGTAGATTTAGAGACGGTTGGGAAATTG	This paper and <a href="#">Alcántara et al., 2021</a>	N/A
dsDNA oligo: crRNA sequence RNaseP target human sample control TAATACGACTCACTATAGGTAATTTCT TACTAAGTGTAGATAAATTAATTGGGTGTGACCCT	<a href="#">Broughton et al.,2020</a>	N/A
ssDNA oligo: primer forward N target SARS-CoV-2 TACAAACATTGGCCGCAAATTGC	This paper and <a href="#">Alcántara et al., 2021</a>	N/A
ssDNA oligo: primer reverse N target SARS-CoV-2 CCAATGCGCGACATTCCG	This paper and <a href="#">Alcántara et al., 2021</a>	N/A
ssDNA oligo: primer forward ORF1ab target SARS-CoV-2 GTTGTTTCAGTTGACTTCGC	This paper and <a href="#">Alcántara et al., 2021</a>	N/A
ssDNA oligo: primer reverse ORF1ab target SARS-CoV-2 GACAATTTCAAGCACAGG	This paper and <a href="#">Alcántara et al., 2021</a>	N/A
ssDNA oligo: primer forward RNaseP target human sample control ACTCAGCCATCCACATCC	This paper and <a href="#">Alcántara et al., 2021</a>	N/A
ssDNA oligo: primer reverse RNaseP target human sample control CACCCTCAATGCAGAGTC	<a href="#">Broughton et al.,2020</a>	N/A
reporter_FQ: /56-FAM/TTATT/3IABkFQ/	<a href="#">Chen et al. (2018)</a>	N/A
<b>Software and algorithms</b>		
ImageJ	<a href="#">Schneider et al., 2012</a>	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>
GraphPad Prism version 9	GraphPad Software, La Jolla, CA	<a href="http://www.graphpad.com">www.graphpad.com</a>
Stata v14.0	StataCorp, College Station, TX	<a href="https://www.stata.com/">https://www.stata.com/</a>
<b>Other</b>		
96-well flat clear bottom black polystyrene TC-treated microplates	Corning®	Cat # 3603

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NanoDrop One microvolume UV-Vis spectrophotometer	Thermo Fisher	Cat # ND-ONE-W
safeVIEW: LED/Blue Light Transilluminator	Cleaver Scientific	Cat # SAFEVIEW
Cytation™ 5 Cell Imaging Multi-Mode Reader	BioTek Instruments	N/A
Synergy™ H1 Hybrid Multi-Mode Reader	BioTek Instruments	N/A

## MATERIALS AND EQUIPMENT (OPTIONAL)

- Buffers:

### 10× CrB Buffer

Stocks	Final concentration	Amount
Tris-HCl (1 M) (pH 7.9, 25°C)	100 mM	2.5 mL
NaCl (1 M)	100 mM	2.5 mL
Nuclease-free water	n/a	20 mL
<b>Total</b>	<b>n/a</b>	<b>25 mL</b>

\*Store at –20°C

### 1× CrB1 Buffer

Stocks	Final concentration	Amount
10× CrB Buffer	1×	3.9 mL
Nuclease-free water	n/a	100 µL
<b>Total</b>	<b>n/a</b>	<b>4 mL</b>

\*Always use fresh working stocks.

### 1× CrB2 Buffer

Stocks	Final concentration	Amount
1× CrB1 Buffer	1×	3340 L
MgCl <sub>2</sub> (1 M)	17.65 mM	60 µL
<b>Total</b>	<b>n/a</b>	<b>3.4 mL</b>

\*Always use fresh stocks.

**Note:** If using locally produced enzymes, prepare the following buffers

### 5× RPB Buffer

Stocks	Final concentration	Amount
Tris-HCl (1 M) (pH 8.4, 25°C)	250 mM	6.25 mL
KCl (1 M)	375 mM	9.375 mL
MgCl <sub>2</sub> (1 M)	15 mM	375 µL
Trehalose dihydrate	50%	12.5 g
DTT (1 M)	50 mM	1.25 mL
EDTA (0.5 M)	0.5 mM	25 µL
Nuclease-free water	n/a	to 25 mL
<b>Total</b>	<b>n/a</b>	<b>25 mL</b>

\*Storage at –20°C.

#### 4× RPB Buffer

Stocks	Final concentration	Amount
5× RPB Buffer	4×	200 μL
10 mM dNTPs mixture	1.6 mM	40 μL
Nuclease-free water	n/a	10 μL
<b>Total</b>	<b>n/a</b>	<b>250 μL</b>

\*Storage at –20°C. Each 250 μL aliquot yields 50 RT-PCR reactions.

#### MB6 buffer

Stocks	Final concentration	Amount
Tris-HCl (1 M) (pH 8.0, 25°C)	50 mM	1.25 mL
NaCl (1 M)	100 mM	2.5 mL
DTT (1 M)	5 mM	125 μL
EDTA (0.5 M)	0.1 mM	5 μL
Triton X-100	0.1%	25 μL
Glycerol	50%	12.5 mL
Nuclease-free water	n/a	8.5 mL
<b>Total</b>	<b>n/a</b>	<b>25 mL</b>

\*Storage at –20°C

- Oligonucleotides: The following primers were used at 5 μM stocks:

**Note:** Dissolve the primers using nuclease-free water to 100 μM concentration. Then dilute to 5 μM in an appropriate volume.

Gene	Orientation	Name	Sequence (5' – 3')	Amplicon Size (bp)
ORF1ab	Forward	FCb3	GTTGTTTCAGTTGACTTCGC	168
	Reverse	RCb2	GACAATTTACAAGCACAGG	
N	Forward	FN1	TACAAACATTGGCCGCAAATTGC	131
	Reverse	RN2	TCATCCAATTTGATGGCACC	
RNaseP	Forward	FR1	ACTCAGCCATCCACATCC	175
	Reverse	RR2	CACCTCAATGCAGAGTC	

- Additional reagents:
  - Working stock 2 mg/mL BSA, dissolved in Nuclease-free water
  - Working stock 1 M MgCl<sub>2</sub>
  - Nuclease-free water
  - 56-FAM/TTATT/3IABkFQ dually-labeled probe should be dissolved at 100 μM concentration in nuclease-free water. HPLC-purified probe is recommended and can be purchased from any oligo synthesis supplier.

**Note:** If using locally produced enzymes, then prepare a working 20× stock of enzymes as follows:

- Enzymes:
  - 20× working stock M-MLV reverse transcriptase: Dilute your enzyme stock in MB6 buffer to a final concentration of 34 ng/μL.
  - 20× working stock Taq DNA polymerase: Dilute your enzyme stock to a final concentration of 32 ng/μL.
- Equipment:

- The protocol described here for SARS-CoV-2 detection can be used in any conventional thermal cycler. Here, we used the T-100 thermal cycler (Bio-Rad) (Cat. No. 1861096) and the portable BentoLab (<https://www.bento.bio/>).
- Fluorescence signal visualization used the microplate reader Synergy H1 (BioTek Instruments) for a quantitative readout, or a transilluminator safeVIEW: LED/Blue Light (Clever Scientific) for a qualitative readout.
- RT-PCR product visualization uses 5% agarose gel electrophoresis. In this protocol, the multi-SUB Choice, Wide Midi Horizontal Electrophoresis System was used (Clever Scientific).

### STEP-BY-STEP METHOD DETAILS

The present protocol describes the procedure for a conventional RT-PCR reaction with locally produced enzymes (Mendoza-Rojas et al., 2021) coupled to CRISPR/Cas12a detection using a fluorescence microplate reader, or direct fluorescence visualization (Alcántara et al., 2021). This protocol is intended to be used for SARS-CoV-2 RNA detection. However, the described methodology could be set up as a base protocol for molecular detection of nucleic acid molecules (RNA/DNA) of any other pathogen or microorganism of interest.

**Note:** In this protocol, we describe RT-PCR and CRISPR/Cas recipes for 32 reactions, to fit in the BentoLab portable system. All the examples in the article correspond to a 32 reactions workflow (28 samples + controls). Scale the required volume of each reagent depending on the total number of samples to test or if a 96-well thermocycler is available.

#### Sample RNA extraction

This protocol has been validated with nasopharyngeal swab samples. Use your locally approved viral RNA extraction or other approved commercial alternative kits. Here, the viral RNA material was extracted using the QIAamp viral RNA Mini kit (QIAGEN).

1. To perform RNA extraction, 200  $\mu$ L of sample or negative extraction control (NEC) was used according to the manufacturer protocol (<https://www.qiagen.com/us/resources/download.aspx?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en>). Proceed with the Spin protocol or Vacuum protocol depending on vacuum pump and manifold availability.
2. Elute RNA with 100  $\mu$ L of nuclease-free water.

**Note:** A negative extraction control (NEC) should be included during the RNA extraction step to evaluate contamination during sample handling. A NEC could be a pool of verified negative patient samples, commercial alternatives (i.e., human DNA in stabilizing synthetic matrix, such as the one available in the HDPCR<sup>TM</sup> SARS-CoV-2 assay (ChromaCode)), or even molecular-grade nuclease-free water.

#### One-step RT-PCR

⌚ **Timing:** two hours from reaction preparation to results

This protocol was standardized to amplify two gene SARS-CoV-2 targets, at the 5'- and 3'-end of the genome, as well as a region of the RNaseP human gene as RNA extraction control using a one-step RT-PCR reaction. Examples used M-MLV reverse transcriptase (RT) and Taq DNA polymerase produced in the laboratory. Commercial enzymes can be used as an alternative.

**Alternatives:** The following commercial kits have been evaluated in replacement of the enzymes mentioned here, M-MLV reverse transcriptase and/or Taq-polymerase. Commercial kits can be used as one- or two-step RT-PCR reactions without interfering with the CRISPR/Cas detection assay. For one-step reactions we tested the 2 $\times$  One-Step RT-PCR Master Mix (Cat. No. 28113, Norgen Biotek). For two-step reactions we tested RevertAid First Strand

cDNA Synthesis Kit (Cat. No. K1622, ThermoFisher) coupled with DreamTaq Green PCR Master Mix (2×) (Cat. No. K1081, ThermoFisher) or Taq DNA polymerase with Standard Taq Buffer (Cat. No. M0273S, NEB). Other commercial enzymes are expected to be suitable for nucleic acid amplification followed by detection with the proposed CRISPR/Cas system described in this protocol.

The one-step RT-PCR reaction produces first a DNA copy (cDNA) of the target RNA molecules extracted from nasopharyngeal swabs or saliva samples. After this reverse transcription step, the cDNA template is amplified by the Taq DNA polymerase to generate dsDNA copies of the target region of interest (Álvarez-Fernández, 2013; Chaubal et al., 2018; Graham et al., 2021; Mahony et al., 2011).

**Note:** Each gene target is amplified individually by RT-PCR.

### 3. Master mix preparation

**△ CRITICAL:** It is strongly recommended to prepare the master mix in a different laboratory area from where clinical samples are manipulated or where the samples are analyzed. Amplicon contamination can severely compromise your results. Follow the general recommendations for molecular analysis of clinical samples (WHO. World Health Organization. (2018))

**▣ Pause point:** Thaw all reagents on ice, while pre-heating a thermoblock or thermocycler at 50°C.

- a. Prepare the master mix according to the following recipe (i.e., scale the required volume of each reagent depending on the total number of samples to test). Here we work with 33 reactions corresponding to a final volume of 660 μL considering a 2 μL sample to be analyzed and a total volume of 20 μL for each reaction.

Reagent	Initial concentration	Final concentration	Volume (μL) per reaction	Volume (μL) for 33 reactions
RPB4x	4x	1x	5	165
M-MLV RT	20x	1x	1	33
Taq polymerase	20x	1x	1	33
Reverse primer	5 μM	0.2 μM	0.8	26.4
Forward primer	5 μM	0.2 μM	0.8	26.4
Nuclease-free water	-	-	9.4	310.2
<b>Final volume</b>			<b>18</b>	<b>594</b>

**Note:** It is recommended to prepare 1-2 additional reactions due to some volume loss by pipetting.

**△ CRITICAL:** We strongly recommend preparing and maintaining the master mix on ice (or use an IsoFreeze® PCR rack) to avoid unspecific product amplification (i.e., primer dimerization) (Graham et al., 2021).

- b. Mix by gently vortexing and spin down for few seconds.
- c. Dispense 18 μL in PCR tubes (0.2 mL). Individual tubes or PCR strips can be used.
- d. Securely close all tubes and spin down the tubes for few seconds.

Place the tubes on ice (or using an IsoFreeze® PCR rack)

**△ CRITICAL:** To avoid amplicon contamination, the addition of RNA samples extracted from clinical samples should be performed in a dedicated laboratory area, pipette set, and BSL2

hood. This area should be restricted to any incoming amplified material. Gloves and coats should also be dedicated to this area.

- Add 2  $\mu\text{L}$  of extracted RNA samples to each reaction (step 2). To evaluate potential reaction contamination and reaction performance, a no template control (NTC), a NEC (step 1), and a positive control should be included in each assay, respectively.

**Note:** For the NTC, nuclease-free water must be added to the respective PCR tube. For the positive control, genomic RNA, RNA standards (e.g., as the ones available in the BEI Resources catalogue (<https://www.beiresources.org/>)) or sample pools from certified positive samples for SARS-CoV-2 can be used.

- Insert the tubes or strips into the thermocycler from the BentoLab system (alternative thermocyclers can be used instead) (Figure 1).

**△ CRITICAL:** Thermocyclers must be in a dedicated area. Ensure that all tubes or strips are sealed properly to avoid cross-contamination between samples and to protect samples from evaporation.

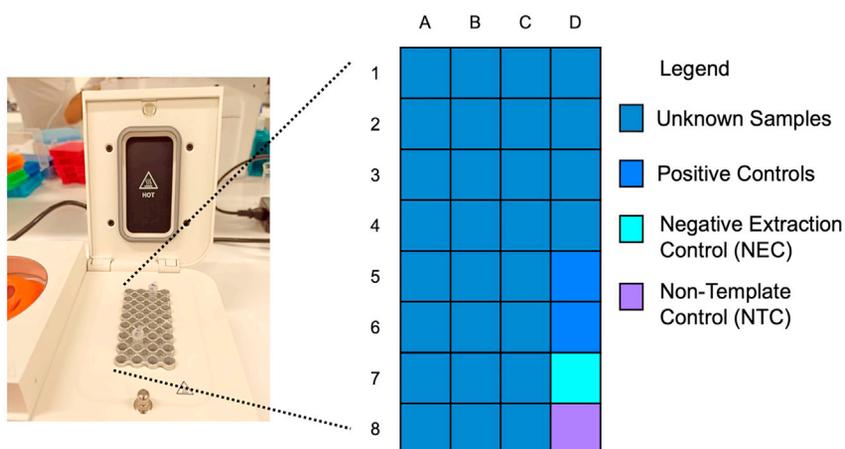
- Run the following RT-PCR program:

PCR cycling conditions			
Step	Temperature	Time	Cycles
Reverse transcription	50°C	20 min	1
Initial denaturation	95°C	5 min	1
Denaturation	95°C	5 s	45 cycles
Annealing/Extension	55°C	30 s	
Hold	10°C	Forever	

**Note:** Total run time 1:40 hours

**⏸ Pause point:** Meanwhile the CRISPR/Cas reaction can be prepared (see CRISPR/Cas reaction below).

- Store the RT-PCR products at 4°C for short term or at  $-20^{\circ}\text{C}$  for mid/long term until the CRISPR/Cas detection assay is performed.



**Figure 1. Recommended plate layout for 32 reactions to be processed in the BentoLab**

**Note:** Optional, the RT-PCR product can be visualized by agarose gel electrophoresis. RT-PCR products are expected to be 168 bp for the ORF1ab target, 131 bp for the N target, and 175 bp for the RNaseP target (Figure 2). If RT-PCR product bands are not visible, see [troubleshooting](#) section – [problem 1](#).

### CRISPR/Cas12a-mediated detection

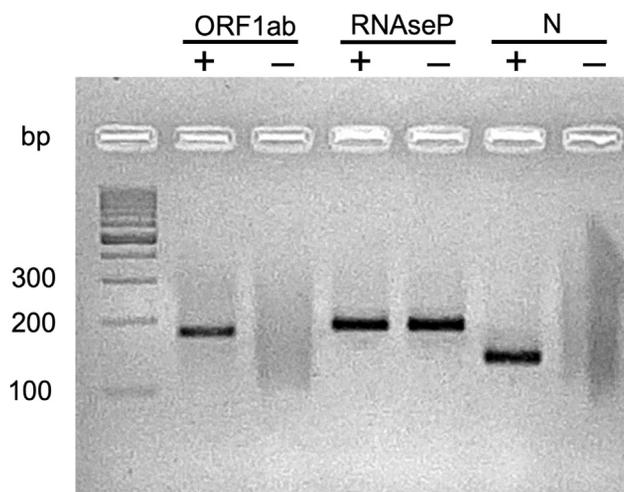
⌚ **Timing:** 35 minutes from reaction preparation to results

This protocol was standardized to detect two *loci* at the 5'- and 3'-end of the SARS-CoV-2 genome, respectively, and a region of the RNaseP human gene. The following crRNA sequences were used for protocol standardization and validation:

Target	Sequence (5' – 3')	length (bp)
ORF1ab	UAAUUUCUACUAAGUGUAGAUUUAGAGACGGUUGGGAAAUUG	42
N	UAAUUUCUACUAAGUGUAGAUCCCCAGCGCUUCAGCGUUC	41
RNaseP	UAAUUUCUACUAAGUGUAGAUAAUUACUUGGGUGUGACCCU	41

The LbCas12a enzyme detects a specific DNA sequence by recognizing the base pairing between crRNA and the target (Chen et al., 2018; Swarts et al., 2017). Upon recognition of the target DNA, Cas12a undergoes structural changes in its RuvC active site that result in an unspecific trans-cleavage activity on any available single-stranded DNA (ssDNA) molecule in the vicinity (Chen et al., 2018; Li et al., 2019). This has been adapted for *in vitro* detection of a target DNA of interest for diagnostic proofs of concept (Broughton et al., 2020; Chen et al., 2018). The CRISPR/Cas12a assay uses a double-labeled reporter probe (i.e., with a fluorophore and quencher) that is cleaved upon Cas12a trans-activity only in the presence of the specific target DNA in the reaction, thereby generating a fluorescent signal that can be detected with a fluorescence plate reader or by direct visualization using a transilluminator (Bonini et al., 2020; Kumar et al., 2020).

**Note:** The following example concentrations and volumes are indicated for the previous 32 RT-PCR reactions for the ORF1ab target that include 28 unknown samples, two



**Figure 2. RT-PCR products from positive and negative controls for ORF1ab, N and RNaseP targets**

The amplification products were run on a 5% agarose gel at 70 V for 60 min. AmpliSize Molecular Ruler (Cat. No. 1708200, Bio-Rad) was used as ladder. Products were visualized using SafeGreen (Cat. No. G108-G, abm) in the loading buffer TriTrack DNA loading dye 6x (Cat. No. R1161, ThermoFisher).

positive controls and two no template controls (NTC). Each target RT-PCR product (ORF1ab, N, and RNaseP) is evaluated individually using the CRISPR/Cas12a assay. An Excel template (Mendeley Data: <https://doi.org/10.17632/8m8z37v8xz.1> Reaction recipe template) is provided to calculate the necessary volume of the required reagents for crRNA re-folding and Cas12a master mix preparation.

**Note:** All buffers required in this segment must be prepared in advance.

8. Thaw all reagents on ice

▣▣ **Pause point:** Pre-heating a thermoblock at 65°C.

9. crRNA re-folding

- Prepare the working stock by diluting the crRNA stock in nuclease-free water to get a final concentration of 1.3 μM (see [before you begin](#), Step 6I, for crRNA production).
- Mix gently by vortexing or tapping, and spin briefly.
- Incubate at 65°C for 10 min.
- Cool at 25°C for 10 min.
- Mix gently by vortexing or tapping. Then spin briefly.

10. 10× Cas12a complex

- Prepare the 10× Cas12a complex according to the following recipe (i.e., scale the required volume of each reagent depending on the total number of samples to test or your stocks' concentration).

Reagent	Initial concentration	Final concentration	Volume (μL) per reaction	Volume (μL) for 33 reactions <sup>a</sup>
Cas12a	5.4 μM	0.1 μM	0.184	6.1
Re-folded crRNA	1.3 μM	0.15 μM	1.164	38.4
Probe	100 μM	3 μM	0.3	9.9
1× CrB1	-	-	8.351	275.6
Final volume	-	-	-	330

<sup>a</sup>Considering one additional reaction due to volume loss by pipetting.

- Protect from light by wrapping the tube with aluminum foil or using dark tubes, if available.
- Incubate the 10× Cas12a complex at 25°C for 10 min.

11. CRISPR/Cas12a reaction preparation

- Each CRISPR/Cas detection reaction is prepared according to the following scheme:

Pipetting Order	Reagent	Initial concentration	Final concentration	Final volume (μL) per assay	Notes
1	CrB2 (17.65 mM MgCl <sub>2</sub> )	1×	1×	85	Mix separately
2	RT-PCR product	-	-	5	
3	Cas12a complex	10×	1×	10	Add right before measuring
	Final volume	-	-	100	

**Note:** CrB2 buffer contains 17.65 mM MgCl<sub>2</sub>, which upon addition of the RT-PCR product and the 10× Cas12a complex brings MgCl<sub>2</sub> to 15 mM.

- Dispense 85 μL of 1× CrB2 in separate tubes, PCR strips, or a 96-well PCR plate.
- Add 5 μL of the RT-PCR product (Step 7).

- d. Mix gently by tapping or using a plate mixer if available. If working with PCR tubes or strips, spin briefly.

**Note:** When working with a multichannel pipette consider increasing the volumes of the first two pipetting steps to avoid inconsistent pipetting. 102  $\mu\text{L}$  CrB2 and 6  $\mu\text{L}$  RT-PCR product for a total of 108  $\mu\text{L}$  from which 90  $\mu\text{L}$  are taken with the multichannel pipette for further analysis.

- e. Dispense 10  $\mu\text{L}$  of the 10 $\times$  Cas12a complex in every well of the 96-well microplate (Step 10c) (Figure 3).
- f. Add 90  $\mu\text{L}$  of the diluted RT-PCR products according to the above plate layout (Step 11c).

Alternative for the Cas12a complex to set up CRISPR/Cas reactions: the EnGen® Lba Cas12a (Cpf1) (Cat. No. M0653S – M0653T, NEB) can be used with the NEB buffer 1 $\times$ .

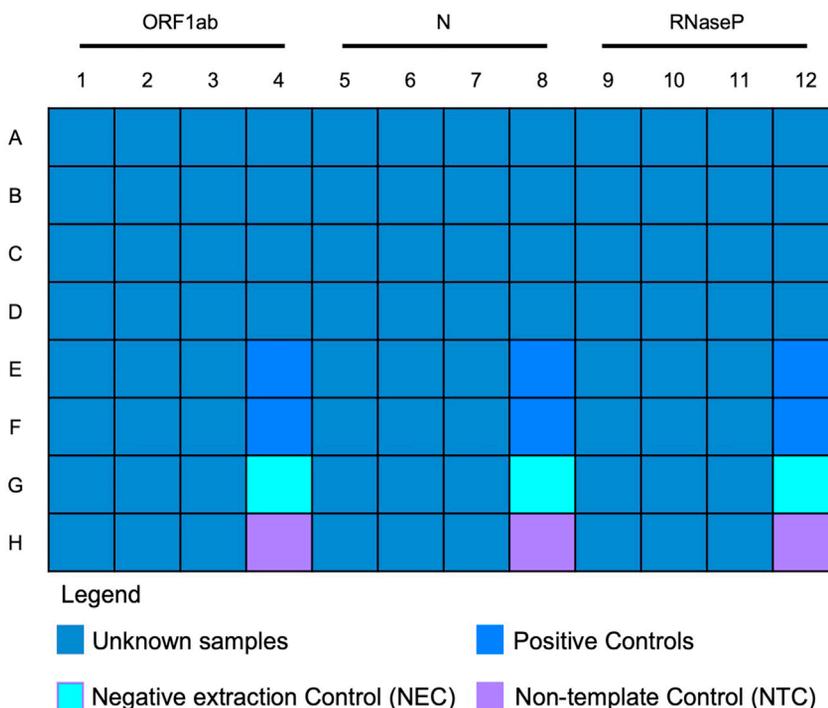
## 12. Outcome readout

A quantitative and a qualitative approach have been evaluated using a microplate reader or by direct fluorescence visualization in microtubes or plates, respectively. The fluorescence is produced by the cleavage of the double-labeled probe (56-FAM/TTATT/3IABkFQ) due to target recognition by the CRISPR/Cas12a system.

- a. Quantitative readout

**Note:** The assay was tested using the Synergy™ H1 Hybrid Multi-Mode microplate reader (BioTek), with software Gen5 v3.10.

- i. Set-up the instrument according to the following parameters:



**Figure 3. Recommended plate layout for CRISPR/Cas reactions in a 96-well microplate**

For each target 32 reactions are included (28 unknown samples + duplicates of positive controls, negative extraction, and no template controls).

General	
Plate type	Select the respective black plate
Temperature	25°C
Kinetic step	
Runtime	35 min
Interval	1 min
Read method <sup>a</sup>	
Detection method	Fluorescence intensity
Read type	Endpoint/Kinetic
Optics type	Monochromators
Excitation (nm)	491
Emission (nm)	525
Gain	85
Read height	7 mm

alf using an equipment that works with a variable bandwidth (i.e., Cytation 5 Multi-Mode reader, Biotek) consider using the following wavelengths: ex  $491 \pm 9$  nm, and em  $525 \pm 21$  nm. If your instrument uses excitation and emission filters, use the corresponding set for Fluorescein (Usually pre-set as GFP)

- ii. Transfer the microplate to the reading instrument and proceed with the measurement.
- b. Qualitative readout

**Note:** The protocol was tested using the transilluminator - safeVIEW: LED/Blue Light (470 nm blue LEDs) (Clever Scientific).

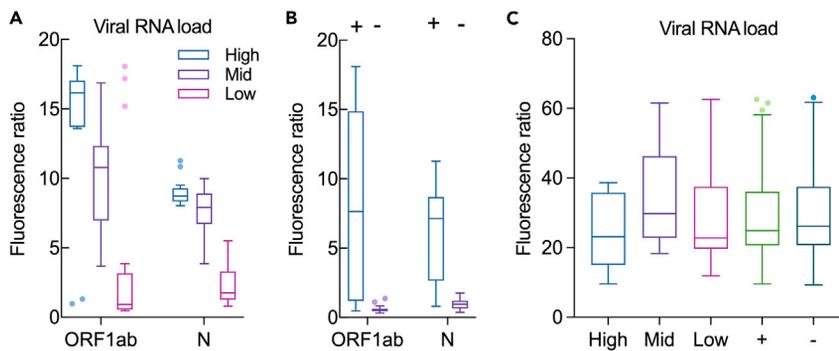
- i. Once the CRISPR/Cas12a reaction is prepared, incubate without light exposure for 30 min.
- ii. Transfer the microplate to the transilluminator and close the filter tap.
- iii. Register the results of direct fluorescence visualization using a photo camera or a smartphone.

**Note:** Using a black cabinet, box, or dark room improves the direct visualization of the results.

## EXPECTED OUTCOMES

Regarding the fluorescence detection in unknown samples, the fluorescence must rapidly increase over time for positive samples. Negative samples and reaction controls do not show a marked increase. However, they can slightly increase over time. An appropriate variable to compare results is the fluorescence ratio between the analyzed sample over the non-template control (NTC) (see the [quantification and statistical analysis](#) section)(Alcántara et al., 2021; Hou et al., 2020). The fluorescence ratio should be higher for positive samples than for the negative ones. There is a positive dependence between fluorescence ratios and the viral RNA load of the sample, higher RNA loads derive in higher fluorescence ratios (Figure 4A). In the validation study with 100 clinical samples, the median fluorescence ratio for positive samples was higher for ORF1ab target (11.4) than the one observed for the N target (7.9) (Figure 4B) (Alcántara et al., 2021). For both detection targets, positive samples show a fluorescence ratio over 4-fold with respect to negative samples. For results interpretation, the fluorescence ratio of the RNaseP human gene must be considered (see below).

The fluorescence signal of the RNaseP gene must increase over time for both SARS-CoV-2 positive and negative clinical samples. Fluorescence ratios of the RNaseP target over the NTC control are expected to be around 25 independently of the viral RNA load (Figure 4C). Fluorescence ratios for the RNaseP target are higher than the ones observed for SARS-CoV-2 targets. In the validation study, the median fluorescence ratio for the 100 tested clinical samples for RNaseP was around 25 (Alcántara et al., 2021). If the fluorescence ratio is less than two, the assay must be repeated to rule out any technical error. If the repeated test fluorescence ratio is less



**Figure 4. Fluorescence ratio distribution for clinical samples in this example**

Data from 50 SARS-CoV-2 positive and 50 negative samples are shown.

(A) Fluorescence distribution for SARS-CoV-2 positive samples sorted by viral RNA load for the ORF1ab and N targets.

(B) Fluorescence distribution for SARS-CoV-2 positive and negative samples for the two targets (ORF1ab, N).

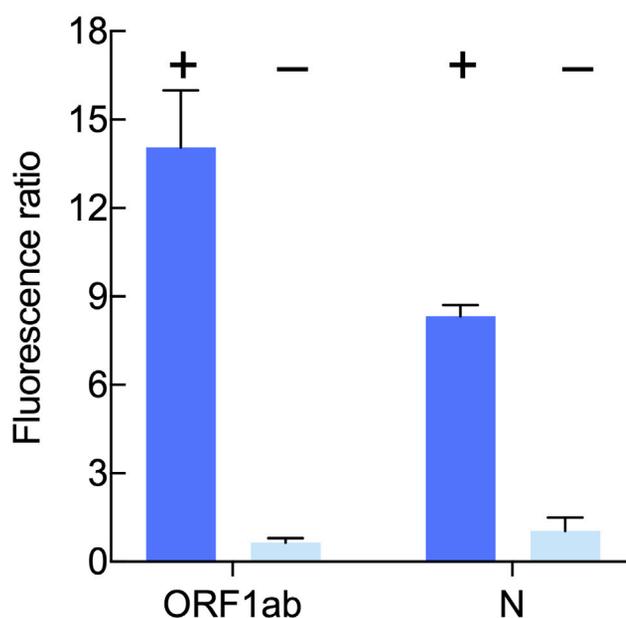
(C) RNaseP fluorescence ratio distribution for SARS-CoV-2 samples, sorted by viral RNA load, positive and, negative controls.

Tukey boxplots in A-C indicate the median, 25%, and 75% percentiles. Whiskers indicate minimum and maximum values excluding outliers (full circles).

than two, the sample quality must be checked, or a new sample should be required (see [troubleshooting](#) section).

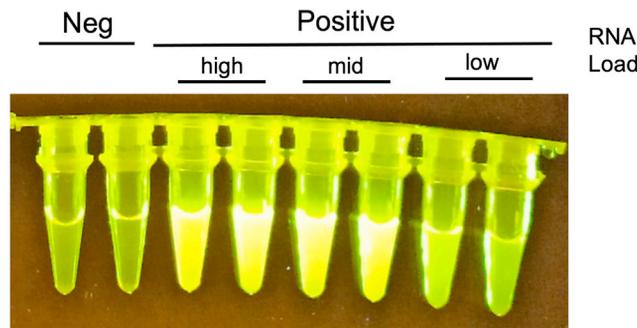
Fluorescence ratios for positive controls must be higher than the negative controls. In the validation study with 100 clinical samples, a mean fluorescence ratio of  $13.96 \pm 1.72$  RFU and  $0.64 \pm 0.14$  RFU were reported for the positive and negative controls for the ORF1ab target, respectively. Likewise, an average fluorescence ratio of  $8.37 \pm 0.35$  RFU and  $1.05 \pm 0.43$  RFU were calculated for the positive and negative controls for the N target, respectively ([Figure 5](#)). If fluorescence is not observed, see the [troubleshooting](#) section – [problems 2](#) and/or [3](#).

Finally, the observation of fluorescence is indicative of a positive result for SARS-CoV-2. Positive samples show a high fluorescence intensity ([Figure 6](#)). If weak fluorescence signal is visualized, see the [troubleshooting](#) section – [problem 4](#).



**Figure 5. Fluorescence ratio for positive and negative controls**

Fluorescence ratio data represent the mean of five independent experiments. Error bars show standard deviation.



**Figure 6. Visual detection of the amplified N target using CRISPR/Cas12a-dependent fluorescence signal**  
RT-PCR products of positive and negative SARS-CoV-2 samples were observed in a blue light LED transilluminator.

## QUANTIFICATION AND STATISTICAL ANALYSIS

In the quantitative approach, the fluorescence ratios over the NTC are evaluated to determine positivity.

**Note:** An Excel template (Mendeley Data: <https://doi.org/10.17632/8m8z37v8xz.1>. Fluorescence ratios calculation template) can be used to calculate the fluorescence ratio of each sample. Sheet 1 is the original readout report and exported as an Excel file from the Biotek instruments. The template must be modified if using a different instrument and software. Sheet 2 shows the normalized fluorescence ratios between the sample and the NTC control.

1. Export the raw data for all samples as an Excel spreadsheet. Relative fluorescence unit (RFU) time courses will appear in one column for each sample/well (Table 1).
2. Normalize the fluorescence signal by subtracting the first value (Time = 0) for each sample (Table 2).
3. Calculate the mean for fluorescence values of the positive control.
4. Calculate the fluorescence ratio for each control and sample using the following equation:

$$\frac{RFU_{30min} \text{ Test sample}}{RFU_{30min} \text{ NTC}}$$

Divide the fluorescence signal (RFU) of test samples (positive control or NEC) by the NTC values, both measured at the timepoint 30 min (Table 3).

5. Determine positivity following the algorithm shown in Figure 7,

**Note:** The method using this protocol showed a Limit of Detection (LoD) of  $10^2$  ge/ $\mu$ L using SARS-CoV-2 genomic RNA (NR-52347, BEI Resources). During the validation process five out of 50 clinical samples showed false negative results. All discordant samples showed Cq values more than 33 (Alcántara *et al.*, 2021). However, Cq value distribution in infected people peaked between 23 – 25 Cq values (Buchan *et al.*, 2020). In that context, the described protocol here should be able to detect all clinical samples.

**Table 1. Example of raw data measured on a plate reader**

Time	Temp.	Raw data					
		Sample Example	Sample Example	Positive control	Positive control	NEC	NTC
		C5	C7	E8	F8	G8	H8
0:00:00	25°C	3255	2323	5298	4210	2388	2182
00:15:00	25°C	11773	3051	24534	21618	3633	3431
00:30:00	25°C	22442	3759	47499	43959	5366	5015

**Table 2. Example of normalized data measured on a plate reader**

Time	Temp.	Raw data					
-	-	Sample Example	Sample Example	Positive control	Positive control	NEC	NTC
Plate wells in File S2		C5	C7	E8	F8	G8	H8
00:15:00	25°C	8518	728	19236	17408	1245	1249
00:30:00	25°C	19187	1436	42201	39749	2978	2833

## LIMITATIONS

This protocol has been validated only with clinical upper-respiratory samples (n = 100) and performed in one country (Peru). Although no mutations have been reported in the SARS-CoV-2 target sequences used in this protocol (ORF1ab and N), the test sensitivity could vary depending on local circulating SARS-CoV-2 strains. The detection targets, used in this protocol, have been selected considering genome regions with low variability. We cannot exclude that false negative results could arise due to future variants of SARS-CoV-2. We recommend conducting a local clinical validation before using it as a laboratory-developed test (LDT) for clinical analysis. Negative results do not exclude COVID-19 disease. Molecular tests are part of the diagnostic algorithms worldwide that include other variables such as symptoms, contact to diagnosed patients, among others. False negative results can be produced by errors during sample manipulation and processing. This protocol is not authorized by any governmental institution yet. A technical guide to validate in-house diagnostic tests can be downloaded from the FIND website (<https://www.finddx.org/reports-and-landscapes/in-house-test-development-for-molecular-detection-of-sars-cov-2-en-fr-pt/>). Other outcome readouts such as lateral flow assay on paper strips have not been validated with this protocol.

## TROUBLESHOOTING

### Problem 1

No amplification for the positive control in RT-PCR (RT\_PCR\_outcome)

The lack of RT-PCR products for the positive control indicates that a technical error has occurred during the preparation of the master mix or the RT-PCR running step. Also, that the quality of the reagents could be compromised. Low or null yield amplification can produce a low fluorescence signal that could be misinterpreted as a negative result.

### Potential solution

Check that the used cycling conditions are correct. Storage conditions of the reagents, enzymes and aliquots of the controls must be checked. Replace the working stock of reagents, enzymes or aliquots of the controls if necessary.

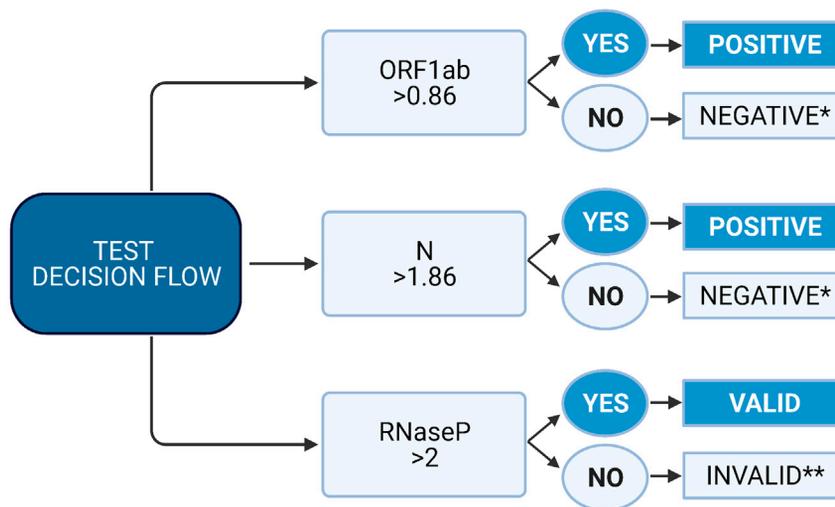
### Problem 2

High fluorescence signal in the no template control and/or negative extraction control (Fluorescence\_outcome)

A high fluorescence signal in the NTC indicates contamination during the CRISPR/Cas reaction or more likely during the amplification step (RT-PCR). A high fluorescence signal in the NTC gives lower fluorescence ratios that could be misinterpreted as negative results in the test samples (false

**Table 3. Example of fluorescence ratio for normalized data measured on a plate reader**

Time	Temp.	Raw data					
-	-	Sample Example	Sample Example	Positive control	Positive control	NEC	NTC
Plate wells in File S2		C5	C7	E8	F8	G8	H8
00:30:00	25°C	6.604	0.494	14.525	13.681	1.025	0.975



**Figure 7. Test decision scheme**

Cut-off values were calculated by a Receiver Operating Characteristic (ROC) curve analysis (Hanley and McNeil, 1982). The selected cut-off values were those that reported the highest percentage of correctly classified samples according to a ROC curve analysis with 100 samples (Alcántara et al., 2021). \* Test again if symptoms occur. If symptoms already present RT-qPCR recommended. \*\* If an invalid result is obtained, see troubleshooting section – problem 5.

negative). In a five-day assay (to assess repeatability), a normalized fluorescence signal average (i.e., time zero subtracted) of  $5171 \pm 821$  RFU (a.u.) and  $2725 \pm 411$  RFU (a.u.) were obtained for both NTC controls of N and ORF1ab targets, respectively, at time point 30 min. Similarly, a normalized fluorescence signal average of  $5350 \pm 234$  RFU (a.u.) and  $1754 \pm 418$  RFU (a.u.) were observed for both NEC control of N and ORF1ab targets, respectively. However, these values may change depending on the instrument used and current setup.

### Potential solution

First, it is recommended to repeat the assay in order to rule out cross contamination between samples or contamination in any reagent used in the CRISPR/Cas reaction. At the same time, it is recommended to run an electrophoresis to visualize any amplicon contamination in the NTC or NEC reaction. If amplicons are observed, contamination in reagents, equipment (i.e., pipettes or tips) or lab area used in the RT-PCR must be considered. Replace the working stock of reagents (i.e., primers, buffer, dNTP, or enzymes), and tips. Clean the pipettes and working areas with a commercial solution such as LookOut® DNA erase spray (Sigma-Aldrich) or 1–2% hypochlorite solution. Cleaning procedures of equipment and lab areas must be considered as part of the working routine.

### Problem 3

Low fluorescence signal in positive controls (Fluorescence\_outcome)

Low fluorescence signal in positive controls could be due to technical errors in the reaction preparation (i.e., miscalculation of reagents or enzymes concentration, improper pipetting dispense, etc.). Also, that the quality of the reagents or enzymes may be compromised. Low fluorescent signals could compromise the analysis of unknown samples, affecting the calculated ratios and the test results. This problem can arise from the RT-PCR step or the CRISPR/Cas-mediated detection. In a five-day assay (to assess repeatability), a normalized fluorescence signal average (i.e., time zero subtracted) of  $43029 \pm 6598.40$  RFU (a.u.) and  $37837.8 \pm 3583.51$  RFU (a.u.) were obtained for both SARS-CoV-2 N and ORF1ab targets, respectively, for a time readout of 30 min. However, the reported values may change depending on the instrument used and/or the reading setup.

#### Potential solution

To rule out a potential lack of RT-PCR amplification, check the reaction products by agarose gel electrophoresis. If RT-PCR products are absent see [problem 1](#). If amplified products are observed, the problem can be solved by focusing on the CRISPR/Cas reaction. Check the reagents and enzyme calculation, equipment setup, and reagents and enzymes storage conditions. Replace the working reagents and/or enzymes stocks if necessary. If a different fluorophore is used in the double-labeled probe, equipment setup must be standardized before evaluating unknown clinical samples.

#### Problem 4

Weak fluorescence during direct visualization readout (Visualization\_outcome)

Naked-eyed detection has been validated using a blue-light transilluminator. High fluorescence signal was reported for high- (Cq < 25) and mid-viral load samples (Cq 25–31) ([Figure 6](#)). Using other transilluminators (e.g., UV-light transilluminator) showed weaker fluorescence signal even for high- and mid- viral load samples.

#### Potential solution

It is recommendable to test direct visual readout for control reactions before testing clinical unknown samples, if using other than a blue-light transilluminator. If available, top filter or dark box can be used to improve direct fluorescence visualization.

#### Problem 5

Invalid results (Interpretation\_outcome)

Invalid results are obtained when the fluorescence signal in the RNaseP target is low, producing fluorescence ratios < 2. Low fluorescence ratios can be obtained if there is a high fluorescence signal in the NTC or a low signal in the reactions with the test samples.

#### Potential solution

Verify the presence of RT-PCR products to rule out an amplification problem by agarose gel electrophoresis. If RT-PCR products are absent see [problem 1](#). If amplified products are observed, the problem can be solved by focusing on the CRISPR/Cas reaction. Repeat the CRISPR/Cas reaction to rule out any technical error during the preparation of the Cas12a master mix. Verify the storage conditions of the Cas12a and crRNA stocks to assure the reagents quality. If the repeated fluorescence ratio result is < 2 or RT-PCR products are not adequately amplified, low sample quality must be considered, and a new sample must be requested.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pohl Milón ([pmilon@upc.pe](mailto:pmilon@upc.pe)).

#### Materials availability

All unique reagents developed in this work can be purchased at any gene synthesis company (i.e., primers and crRNA templates).

#### Data and code availability

Supplemental data files have been deposited to Mendeley Data: <https://doi.org/10.17632/8m8z37v8xz.1>.

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100878>.

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### AUTHOR CONTRIBUTIONS

Conceptualization and methodology, R.A., K.P., V.A., and P.M.; investigation, R.A., K.P., J.A.N., G.M.R., E.D., and D.A.; formal analysis, R.A., K.P., J.A.N., and P.M.; writing – original draft, supervision, and visualization, R.A., K.P., and P.M.; writing – review & editing, R.A., K.P., J.A.N., G.M.R., E.D., D.A., V.A., and P.M.; project administration, P.M.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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