A protocol for rapid detection of the 2019 novel coronavirus SARS-CoV-2 using CRISPR diagnostics: SARS-CoV-2 DETECTR

v1: February 15, 2020
v2: February 18, 2020: contact email provided, corrected RNase P gRNA sequence, updates to test interpretation matrix, corrections to LoD and assay reaction time for CDC SARS-CoV-2 qRT-PCR assay

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Conflicts of Interest: JPB, CLF, JS and JSC are employees of Mammoth Biosciences, CYC is on the Scientific Advisory Board of Mammoth Biosciences, and JSC is a co-founder of Mammoth Biosciences. JPB, CLF, JS, CYC and JSC are co-inventors on CRISPR-related technologies.

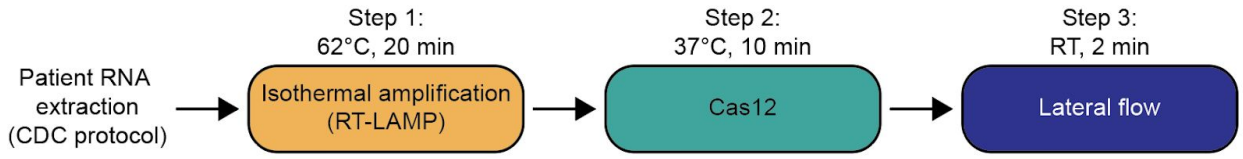
Please contact diagnostics@mammothbiosci.com with any questions or feedback.

*****DISCLAIMER: This protocol has not been approved by the FDA and should not be used as a clinical diagnostic*****

Introduction

Given the global health emergency, rapid transmission, and severe respiratory disease associated with the outbreak of the 2019 novel coronavirus (SARS-CoV-2), Mammoth Biosciences has reconfigured our DETECTR platform to rapidly and accurately detect SARS-CoV-2 using a visual lateral flow strip format within 30 minutes from sample to result. To ensure specificity of detection, we selected a high-fidelity CRISPR detection enzyme and designed sets of gRNAs that can either 1) differentiate SARS-CoV-2 or 2) provide multi-coronavirus strain detection. SARS-CoV-2 DETECTR couples CRISPR detection with isothermal pre-amplification using primers based on protocols validated by the US Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO). Currently in the United States, the CDC SARS-CoV-2 real-time RT-PCR diagnostic panel has a laboratory turnaround time of approximately 4-6 hours, with results that can be delayed for >24 hours after sample collection due to shipping requirements. In addition, these tests are only available in CDC-designated public health laboratories certified to perform high-complexity testing.

Mammoth is working to enable point of care testing (POCT) solutions that can be deployed in areas at greatest risk of transmitting SARS-CoV-2 infection, including airports, emergency departments, and local community hospitals, particularly in low-resource countries. Leveraging an "off-the-shelf" strategy to enable practical solutions within a short time frame, we describe here a protocol that is fast (<30 min), practical (available immediately from international suppliers), and validated using contrived samples.



Specifications	
Targets	• N-gene (SARS-CoV-2 specific) • E-gene (SARS-CoV, bat-SARS-like-CoV, and SARS-CoV-2 coronaviruses) • RNase P (human sample control)
Limit of detection	70-300 copies/µl input

Table 1 | SARS-CoV-2 DETECTR assay workflow and specifications.

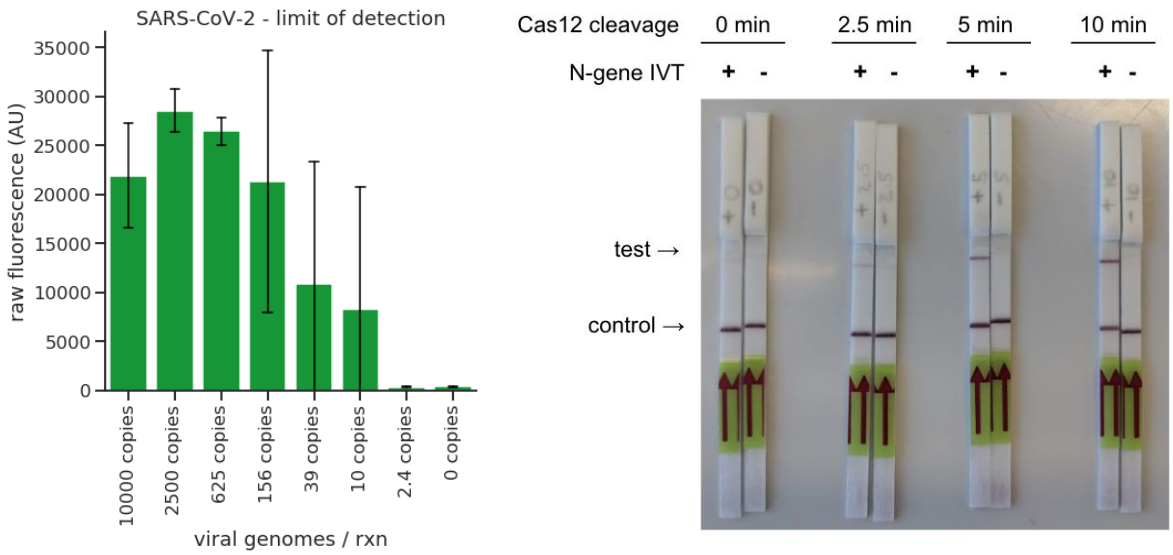


Figure 1 | SARS-CoV-2 DETECTR has a limit of detection (n=7) of 156-625 copies per 20 µl reaction (or 70-300 copies per µl input) and generates a clear visible signal on lateral flow strips within 30 minutes sample to result.

SARS-CoV-2 DETECTR Reagents

Step 1: Isothermal amplification (62°C, 20 min)
RT-LAMP Master Mix (Supplier: NEB)
DNA oligos (Supplier: IDT)

Primer sequences:

Name	Sequence (5' → 3')
N-gene F3	AACACAAGCTTTCGGCAG
N-gene B3	GAAATTTGGATCTTTGTCATCC
N-gene FIP	TGCGGCCAATGTTTGTAATCAGCCAAGGAAATTTTGGGGAC
N-gene BIP	CGCATTGGCATGGAAGTCACTTTGATGGCACCTGTGTAG
N-gene LF	TTCCTTGTCTGATTAGTTC
N-gene LB	ACCTTCGGGAACGTGGTT
E-gene F3	CCGACGACGACTACTAGC

E-gene B3	AGAGTAAACGTAAAAAGAAGGTT
E-gene FIP	ACCTGTCTCTTCCGAAACGAATTTGTAAGCACAAGCTGATG
E-gene BIP	CTAGCCATCCTTACTGCGCTACTCACGTTAACAATATTGCA
E-gene LF	TCGATTGTGTGCGTACTGC
E-gene LB	TGAGTACATAAGTTCGTAC
RNaseP POP7 F3*	TTGATGAGCTGGAGCCA
RNaseP POP7 B3*	CACCCTCAATGCAGAGTC
RNaseP POP7 FIP*	GTGTGACCCTGAAGACTCGGTTTTAGCCACTGACTCGGATC
RNaseP POP7 BIP*	CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCTGGTC
RNaseP POP7 LF*	ATGTGGATGGCTGAGTTGTT
RNaseP POP7 LB*	CATGCTGAGTACTGGACCTC

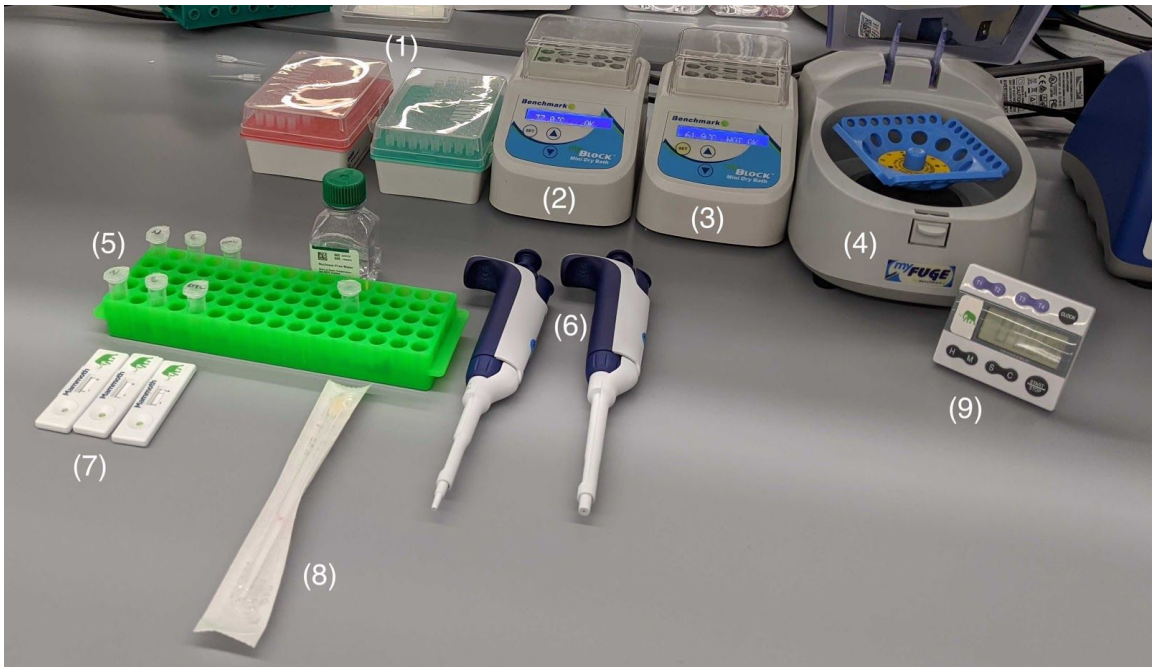
* RNaseP POP7 primers published in Curtis *et al.*, (2018).

Step 2: Cas12 detection (37°C, 10 min)
LbCas12a (Supplier: NEB)
crRNA (Supplier: Synthego)
Reporter (Supplier: IDT)

Name	Sequence (5' → 3')
N gene gRNA (SARS-CoV-2 specific)	UAAUUUCUACUAAGUGUAGAUCCCCCAGCGCUUCAGCGUUC
E gene gRNA (pan-coronavirus)	UAAUUUCUACUAAGUGUAGAUGUGGUAUUCUUGCUAGUUAC
RNase P gRNA (Sample control)	UAAUUUCUACUAAGUGUAGAUAAUUACUUGGGUGUGACCCU
Reporter	/56-FAM/TTATTATT/3Bio/

Step 3: Lateral flow (RT, 2 min)
Milenia HybriDetect 1 lateral flow strips (Supplier: TwistDx)

Sample equipment



(1) Pipette tips
(2) 37°C heat block
(3) 62°C heat block
(4) Microcentrifuge
(5) Eppendorf tubes
(6) Pipettes
(7) Lateral flow strips
(8) Sample collection device (nasopharyngeal swab)
(9) Timer

Experimental Protocol

A. Prepare nucleic acid sample and CRISPR reagents

1. Extract patient RNA following CDC recommendations.

2. Prepare LbCas12a RNP complexes for the samples to be tested. One complex for N-gene, E-gene, and RNase P gRNAs is needed for each sample.

Reagent	Volume	Final Concentration
Nuclease-free water	15.75 µL	
10X NEBuffer 2.1	2 µL	1X
1 µM LbCas12a	1 µL	50 nM
1 µM gRNA	1.25 µL	62.5 nM
TOTAL VOLUME	**20 µL**	

3. Incubate LbCas12a with gRNA to generate RNP complexes for 30 minutes at 37°C.

4. Add reporter substrate to final concentration of 500 nM.

5. Place reactions on ice until ready to proceed.
    a. Complexes are stable at 4°C for at least 24 hours.

B. Run DETECTR reaction

1. On ice, prepare three RT-LAMP reactions, one each for N-gene, E-gene, and RNase P primer sets:

Reagent	Volume	Final Concentration
10X Isothermal Amplification Buffer (NEB)	2.5 µL	
100 mM $MgSO_4$ (NEB)	1.13 µL	6.5 mM (4.5 mM added, 2 mM in 1X IsoAmp Buffer)
10 mM dNTPs (NEB)	3.5 µL	1.4 mM
10X Primer Mix	2.5 µL	0.2 µM F3 0.2 µM B3 1.6 µM FIP 1.6 µM BIP 0.8 µM LF 0.8 µM LB
Bst 2.0 polymerase (NEB)	1 µL	8 units / rxn
Warmstart RTx (NEB)	0.5 µL	7.5 units / rxn
Nuclease-free water	3.87 µL	

Nucleic acid sample	5 µL	
TOTAL VOLUME	**25 µL**	

2. Incubate at 62°C for 20 minutes.
    a. Note: Use precaution when opening amplification tubes to prevent amplicon contamination.

3. Combine 2 µL of the RT-LAMP reaction with 20 µL of the LbCas12a RNP complex with the appropriate gRNA.

4. Add 80 µL of 1X NEBuffer 2.1.

5. Incubate at 37°C for 10 minutes.

6. Insert Milenia HybriDetect 1 (TwistDx) lateral flow strip directly into reaction.

7. Allow the lateral flow strip to run for 2 minutes at room temperature and observe the result.

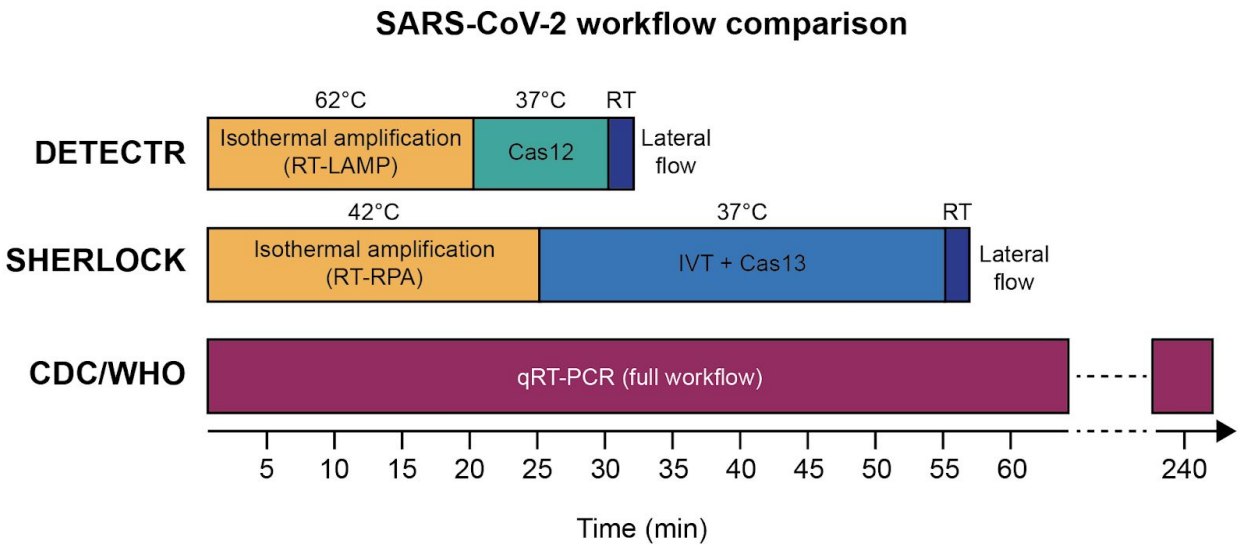
C. Test interpretation

Note: The line closest to the sample pad is the control line and the line that appears farthest from the sample pad is the test line (see Figure 1). A sample with complete cleavage of the reporter molecule may appear to have no signal at the control line.

N-gene	E-gene	RNase P	Interpretation
+	+	+	SARS-CoV-2 positive
+	+	-	Error
+	-	-	Error
+	-	+	Indeterminate
-	+	+	Indeterminate
-	-	+	SARS-CoV-2 negative
-	-	-	Error

Appendix

While we were preparing this white paper, another protocol for SARS-CoV-2 detection using CRISPR diagnostics (SHERLOCK, v.20200214) was published. We compare the assay workflows and specifications between CRISPR diagnostics and established CDC/WHO protocols below. (Note: as of this publication, CRISPR diagnostics workflows have not yet been approved by the FDA)



Appendix Figure 1 | Comparison of SARS-CoV-2 assay workflows for DETECTR, SHERLOCK, and CDC/WHO.

	SARS-CoV-2 DETECTR	SARS-CoV-2 SHERLOCK	CDC SARS-CoV2 qRT-PCR
Target	N gene & E gene (N gene gRNA compatible with CDC N2 amplicon, E gene compatible with WHO protocol)	S gene & Orf1ab gene	N-gene (3 amplicons)
Sample control	RNase P	None	RNase P
Limit of Detection	70-300 copies/µl input	10-100 copies/µl input	3.16-10 copies/µL input
Assay reaction	~30 min	~60 min	~120 minutes

time			
Assay components	RT-LAMP (62°C, 20 min) Cas12 (37°C, 10 min) Lateral flow (RT, 2 min)	RT-RPA (42°C, 25 min) IVT + Cas13 (37°C, 30 min) Lateral flow (RT, 2 min)	UDG digestion (25°C, 2 min), reverse transcription (50°C, 15 min), denature (95°C, 2 min) amplification, (95°C, 3 sec; 55°C 30 sec; 45 cycles)
Heavy instrumentation required	No	No	Yes
FDA EUA approval	No	No	Yes

Appendix Table 1 | Comparison of SARS-CoV-2 specifications for CRISPR diagnostic protocols to the current CDC assay.