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A protocol for rapid detection of the 2019 novel coronavirus SARS-CoV-2 using CRISPR diagnostics: SARS-CoV-2 DETECTR v.3

Mammoth Biosciences[1], James P. Broughton[2], Wayne Deng[3,4], Clare L. Fasching[2], Jasmeet Singh[2], Charles Y. Chiu[3,4,5], Janice S. Chen[2]

[1][Mammoth Biosciences, Inc., South San Francisco, California, USA], [2]Mammoth Biosciences, Inc., South San Francisco, California, USA, [3]Department of Laboratory Medicine, University of California, San Francisco, California, USA, [4]UCSF-Abbott Viral Diagnostics and Discovery Center, San Francisco, California, USA, [5]Department of Medicine, Division of Infectious Diseases, University of California, San Francisco, California, USA

1 Works for me dx.doi.org/10.17504/protocols.io.bfhcjj2w

Mammoth Biosciences

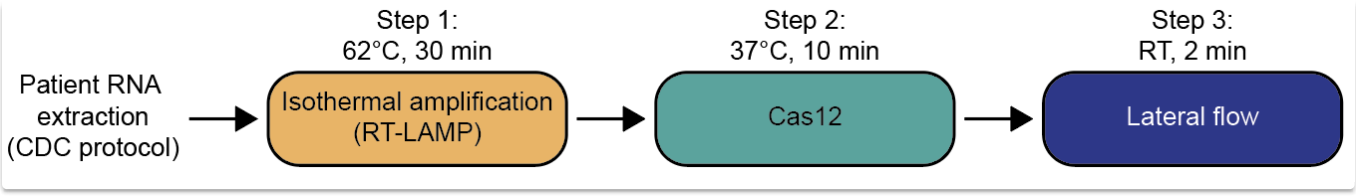
ABSTRACT

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	10 copies/µl input

Citation: Mammoth Biosciences, James P. Broughton, Wayne Deng, Clare L. Fasching, Jasmeet Singh, Charles Y. Chiu, Janice S. Chen (04/23/2020). A protocol for rapid detection of the 2019 novel coronavirus SARS-CoV-2 using CRISPR diagnostics: SARS-CoV-2 DETECTR. https://dx.doi.org/10.17504/protocols.io.bfhcjj2w

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

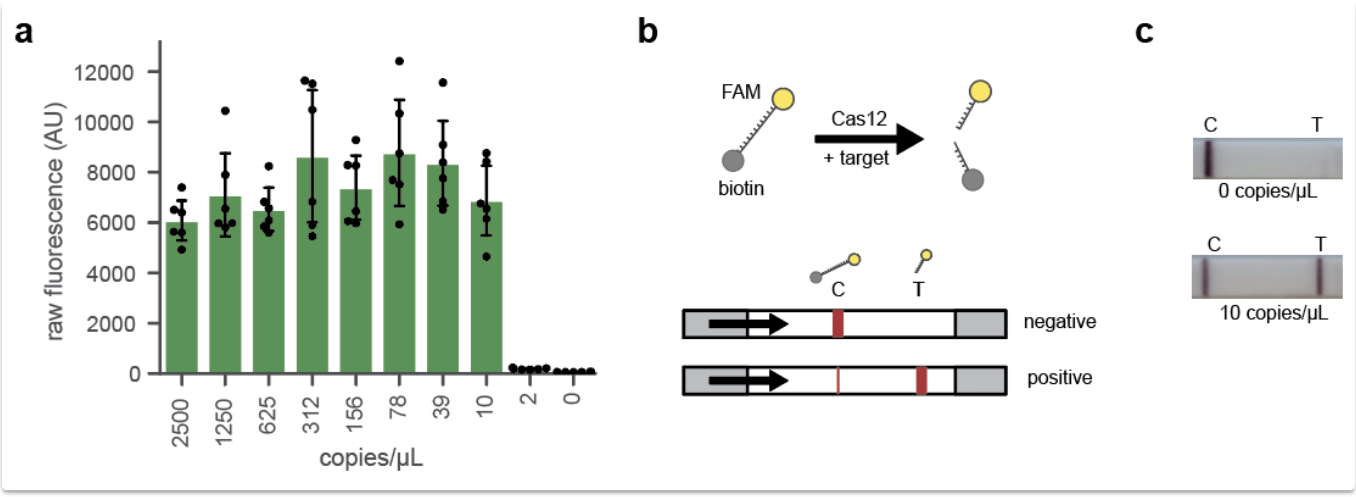


Figure 1 | a) SARS-CoV-2 DETECTR has a limit of detection (n=6) of 10 copies per µl input. **b)** Schematic of DETECTR assay coupled to lateral flow strip. **c)** Representative lateral flow results for the assay shown for 0 copies per µl and 10 copies per µl

Acknowledgements: We thank Vikram Joshi, Nefeli Tsaloglou and Xin Miao for advice and helpful discussions in the preparation of this whitepaper.

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.

EXTERNAL LINK

https://mammoth.bio/2020/02/15/white-paper-a-protocol-for-rapid-detection-of-sars-cov-2-using-crispr-sars-cov-2-detectr/

ATTACHMENTS

SARS-CoV-2.pdf

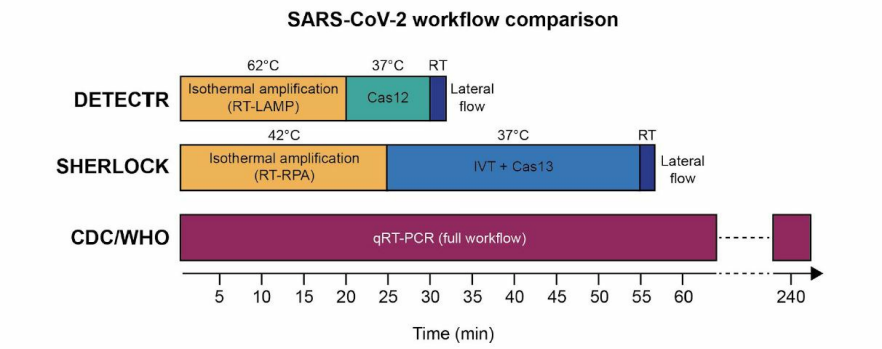
A protocol for rapid detection of SARS-CoV-2 using CRISPR diagnostics_v2.pdf

GUIDELINES

Appendix

While we were preparing this whitepaper, 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)

Citation: Mammoth Biosciences, James P. Broughton, Wayne Deng, Clare L. Fasching, Jasmeet Singh, Charles Y. Chiu, Janice S. Chen (04/23/2020). A protocol for rapid detection of the 2019 novel coronavirus SARS-CoV-2 using CRISPR diagnostics: SARS-CoV-2 DETECTR. https://dx.doi.org/10.17504/protocols.io.bfhcjj2w



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	1 copy/µL input
Assay reaction time	~30 min	~60 min	~45-60 minutes
Assay components	RT-LAMP (62 °C, 290 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 min; 55 °C 30 sec; 45 cycles)
Heavy instrumentation required	No	No	Yes
FDA EUA approval	No	No	Yes

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

MATERIALS TEXT

SARS-CoV-2 DETECTR Reagents

Step 1: Isothermal amplification (62°C, 20 min)

- RT-LAMP Master Mix (Supplier: NEB)

WarmStart LAMP Kit (DNA and RNA) - 100 rxns
by New England Biolabs
Catalog #: E1700S

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- 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*	CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCT GGTC
RNaseP POP7 LF*	ATGTGGATGGCTGAGTTGTT
RNaseP POP7 LB*	CATGCTGAGTACTGGACCTC

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

Curtis KA, Morrison D, Rudolph DL, Shankar A, Bloomfield LSP, Switzer WM, Owen SM (2018). A multiplexed RT-LAMP assay for detection of group M HIV-1 in plasma or whole blood.. Journal of virological methods.
https://doi.org/10.1016/j.jviromet.2018.02.012

Step 2: Cas12 detection (37°C, 10 min)

- LbCas12a (Supplier: NEB)

EnGen Lba Cas12a (Cpf1) - 70 pmol
by New England Biolabs
Catalog #: M0653S

- 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)	UAAUUUCUACUAAGUGUAGAUGACCUGCGAGCGGGUUCUGA

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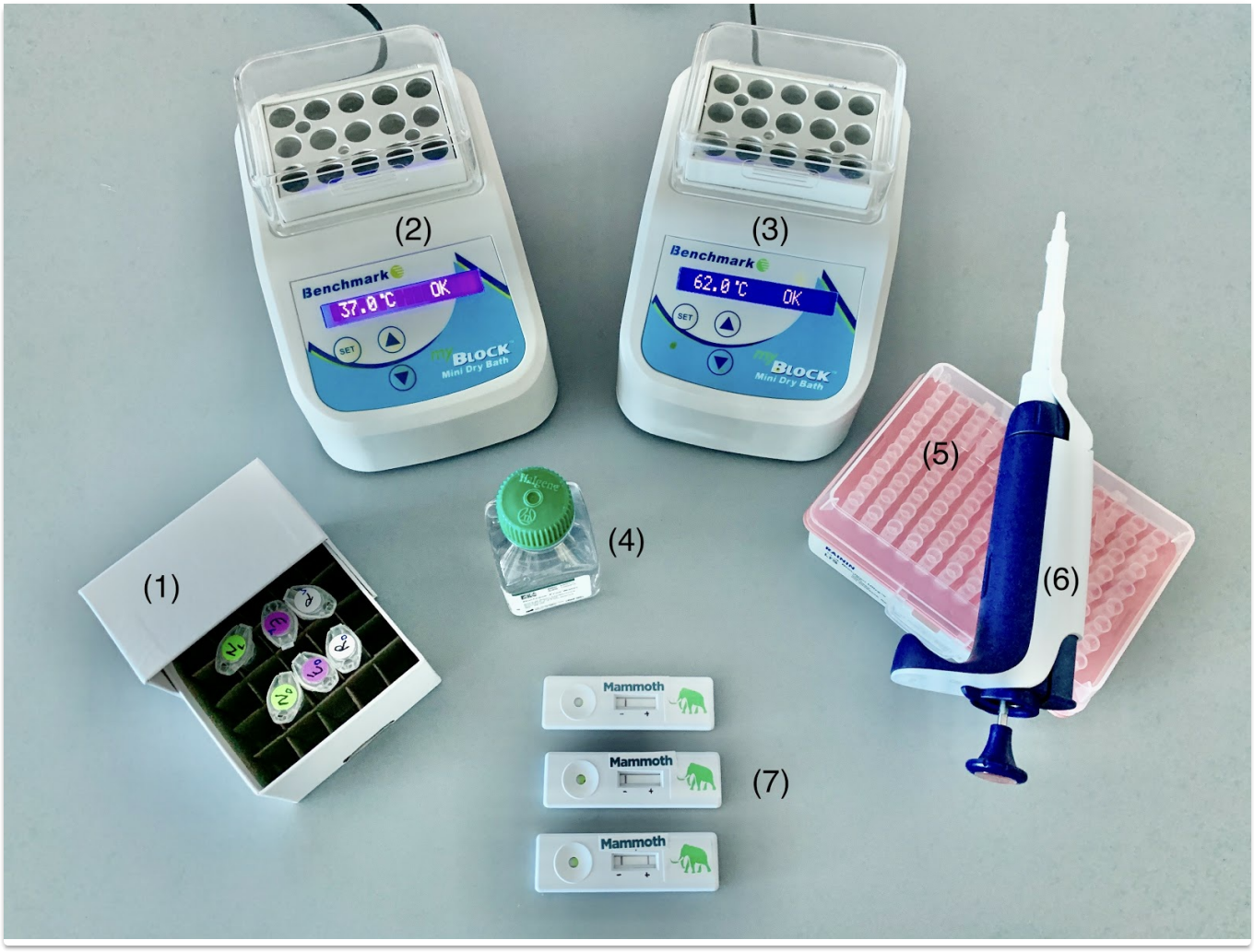
Reporter	/56-FAM/TTATTATT/3Bio/

Step 3: Lateral flow (RT, 2 min)

Milenia HybriDetect 1 lateral flow strips (Supplier: TwistDx

Minimum sample equipment



Sample Equipment

- Pipette tips
- 37 °C heat block
- 62°C heat block
- Microcentrifuge
- Eppendorf tubes
- Pipettes
- Lateral flow strips
- Sample collection device (nasopharyngeal swab)
- Timer

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SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

BEFORE STARTING

Amplicon Contamindation Risk Mitigation

Three approaches of decreasing the risk of amplicon contamination include physical (using hoods), chemical (using DNAzap) and procedural (workflow, PPE, GLP) strategies. We recommend three levels of control: 1. Separate locations for pre- and post-amplification activities, 2. Separate environmental control between the rooms and 3. Separate equipment and personnel. This standard operating procedure (SOP) will provide detailed guidelines that will minimize the risk of amplicon contamination.

Directional workflow (Personnel, reagents, equipment)

The ideal workflow moves from the pre-amp to the post-amp area.

Personnel:

1. One-way directional workflow is critical to minimize carrying amplicons to less contaminated and amplicon-free (pre-amp room) zones.
2. If possible, operators should exclusively work in the pre-amp or post-amp room on any given shift.
3. If only one operator is available, it is recommended that the operator do all amplification reactions first, then proceed to the DETECTR reactions.
4. Proper PPE should be worn to minimize amplicon movement on clothing.
5. Immediately don a disposable lab coat when entering the pre-amp room or prior to working in the post-amp area and dispose of the lab coat prior to leaving the pre-amp room.
6. Immediately don a disposable lab coat when entering the post-amp room or prior to working in the post-amp area and dispose of the lab coat prior to leaving the post-amp room.

Reagents:

1. Ensure your reagents also have a one-way directional flow from receiving/main lab to the pre-amp room.
2. Reconstitute all oligos in the pre-amp room.
3. Make aliquots of primers and reagents to keep in the pre-amp freezer.
4. Amplification reagents may be stored in the pre-amp room in the 4oC fridge or the -20oC freezer.

Equipment:

1. If possible use dead air boxes or hoods for pre-amp and post-amp processes.
2. All equipment found in the pre-amp stays in the pre-amp area.
3. All equipment found in the post-amp stays in the post-amp area.

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**	

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3 Incubate LbCas12a with gRNA to generate RNP complexes for **00:30:00** at **37 °C** .

4 Add *reporter substrate* to final concentration of **500 Nanomolar (nM)** .

5 Place reactions **On ice** until ready to proceed.

Complexes are stable at 4°C for at least 24 hours.

Run DETECTR reaction

6 **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**	

7 Incubate at **62 °C** for **00:30:00** .

Note: Use precaution when opening amplification tubes to prevent amplicon contamination.

8 Combine **2 µl** of the *RT-LAMP reaction* with **18 µl** of the *LbCas12a RNP complex* with the appropriate gRNA.

9 Add **80 µl 1X NEBuffer 2.1** .

10 Incubate at **37 °C** for **00:10:00** .

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

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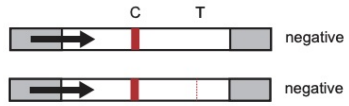
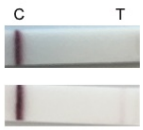
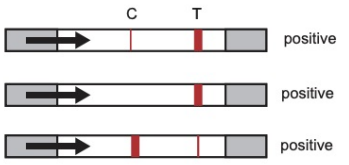
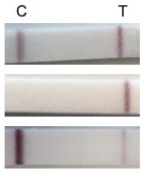
12 Allow the lateral flow strip to run for **00:02:00** at **Room temperature** and observe the result.

Test interpretation

13

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.

Schematic	Example	Description
		Negative strips have no signal at the test line. Negative strips that have been allowed to sit at room temperature for over 10 minutes may display a faint signal at the test line or at the tape seam, but this signal is much fainter than a true positive signal.
		For strips indicating a positive result, there will be an easy to see band at the test line. The control band may or may not be present. The control band is not present when there is complete digestion of the reporter molecule by Cas12. In some cases the test band will be weaker than the control band.

N-gene	E-gene	RNase P	Interpretation
+	+	+/-	SARS-CoV-2 positive
+	-	+/-	Indeterminate
-	+	+/-	Indeterminate
-	-	+	SARS-CoV-2 negative
-	-	-	QC failure

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