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Fluorescent-Reporter Based Assay

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ABSTRACT

The CRISPR-Enhance SARS-CoV-2 detection kit has been designed to detect fragments of the Nucleocapsid ("N") gene and Envelope gene (E) of SARS-CoV-2. An included third target is the human RNase P POP7 gene ("RP") which serves as a control for the extraction of the clinical sample in the absence of a positive SARS-CoV-2 result. Amplification can be performed using a heat block, and CRISPR complex activation and reporter cleavage can be run in a standard microplate reader capable of fluorescence detection. The entire reaction from RT-LAMP amplification to CRISPR-based detection of the target analytes can be performed in approximately one hour.

The CRISPR-Enhance kit comprises of two steps. Step one is a reverse transcriptase loop-mediated amplification (RT-LAMP) where targeted SARS-CoV-2 genomic RNA is reverse transcribed to DNA, and this DNA is amplified by a strand-displacing DNA polymerase. Step two is the transcription of the amplified DNA to activate the collateral cleavage activity of a CRISPR complex programmed to the target RNA sequence. This collateral activity results in cleavage of nucleic acid reporters, resulting in a fluorescent readout detected by a plate reader.

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KEYWORDS

CRISPR, SARS-CoV-2, COVID-19 Diagnostic

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GUIDELINES

All procedures should be performed in a BSL2 laboratory, and specimens should be handled within a Biological Safety Cabinet. All necessary safety precautions should be taken according to the Laboratory guidelines. Precautions must also be taken to prevent cross-contamination of samples.

MATERIALS

NAME	CATALOG #	VENDOR

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QuickExtract™ RNA Extraction Kit	QER090150	Lucigen
WarmStart®Colorimetric LAMP 2X Master Mix with UDG (Cat.No. M1804S)	M1804S	New England Biolabs

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SAFETY WARNINGS

1. Handle all infectious samples with appropriate CDC approved methods
2. Wear appropriate PPE such as lab coats, gloves, N95 respirators, safety goggles etc when handling infectious samples
3. Discard all biohazard waste appropriately
4. Clean all work surfaces with bleach and IPA after use

Nucleic Acid Extraction 18m

1 18m

- The CRISPR-Enhance SARS-CoV-2 detection kit uses QuickExtract™ RNA Extraction Kit

QuickExtract™ RNA Extraction Kit
by Lucigen
Catalog #: QER090150

- Add **10 µl** of patient sample to **10 µl** of pre-aliquoted QuickExtract solution.
- Heat the above mixture at **65 °C** for **00:15:00** followed by **98 °C** for **00:03:00** .

RT-LAMP Master Mix Preparation

2 - Label a new **1.5 mL** microcentrifuge tube for each target (N, E and RP) and prepare a RT-LAMP Master Mix consisting of the WarmStart®Colorimetric LAMP 2X Master Mix with UDG.

WarmStart®Colorimetric LAMP 2X Master Mix with UDG (Cat.No. M1804S)
by New England Biolabs
Catalog #: M1804S

and the appropriate 10x Primer Mix using the recipe in Table 1 below. Make enough of each master mix for all samples to be tested and the necessary controls for each run.

Reagent Name	Volume per reaction	Total Volume
WarmStart®Colorimetric LAMP 2X Master Mix with UDG	12.5 µL	12.5 µL x (N+1)
10x Primer Mix (N, E or RP)	2.5 µL	2.5 µL x (N+1)
RNAse-Free Water	5 µL	5 µL x (N+1)
Total Volume	**20 µL**	**20 µL x (N+1)**

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Table 1: Target Specific RT-LAMP Master Mix Recipe

N = number of extracted samples plus number of controls. Prepare enough for 1 extra (N + 1) sample to allow for overage during reaction set-up.

RT-LAMP Amplification

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- Label a strip tube (**0.2 mL**) with the target name (e.g. N) and strip number corresponding to each sample.
- Add **20 µl** of the RT-LAMP Master Mix from the previous step into one well for each sample and control to be amplified. Repeat for the remaining 2 targets using a new strip for each target (e.g. E or RP)
- Add **5 µl** of extracted RNA in each respective strip tube containing the RT-LAMP Master mix. Vortex the strip tube for **00:00:03** and spin down for **00:00:03** in microcentrifuge with a **0.2 mL** tube adaptor.

Reagent	Volume per reaction
RT-LAMP Master Mix	20 µL
RNA Sample or Controls	5 µL
Total Volume	**25 µL**

Table 2: RT-LAMP Assay Components and reaction volume

- Heat the mixture at **65 °C** for **00:40:00**

CRISPR-Cas Reaction Preparation

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- Preheat a fluorescence microplate reader to **37 °C** .
- For each target tested label a **1.5 mL** tube with the target name (e.g. N, E or RNASE-P) and “Cas Mix”. Prepare a CRISPR Cas Master Mix using the following recipe in Table 3 below, scaling as required for the number of assays to be run (one Cas assay for every RT-LAMP reaction).
- Incubate the mixture at **37 °C** for **00:15:00**
- Pulse vortex for **00:00:03** and spin down for **00:00:03** in a microcentrifuge after all components are added.

Reagent Name	Volume per Reaction	Volume Total
NEB 2.1 Buffer	1.2 µL	1.2 µL x (N+1)
3 µM crRNA (N or E or RP)	0.8 µL	0.8 µL x (N+1)
3 µM lbCas12a	0.4 µL	0.4 µL x (N+1)
RNAse-Free Water	9.6 µL	9.6 µL x (N+1)
Total Volume	12 µL	12 µL x (N+1)

Table 3: Target CRISPR Cas Master Mix Recipe

N = number of extracted samples plus number of controls. Prepare enough for 1 extra (N + 1) sample to allow for overage during reaction set-up.

- In a separate **1.5 mL** microcentrifuge tube prepare the fluorecence reporter as per Table 4

Reagent Name	Volume per Reaction	Volume Total
FAM-FQ	0.2 µL	0.2 µL x (N*+1)
RNAse-Free Water	25.8 µL	25.8 µL x (N*+1)
Total Volume	12 µL	12 µL x (N*+1)

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Table 4: Flourescence Reporter Mix Recipe

N = number of extracted samples multiplied by the total number of genes plus number of controls. Prepare enough for 1 extra (N* + 1) sample to allow for overage during reaction set-up*

CRISPR-Cas Detection

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- Add 26 µL of the Fluorescent reporter Mix made in step 4 to each well of a 384 well-plate corresponding to the number of samples and controls for every gene (N, E or RP).
- Add 2 µL of the RT-LAMP product from step 3 to each well containing the fluorescent reporter.
- Add 12 µL of the CRISPR-Cas master mix to the wells containing the corresponding RT-Lamp product and fluorescent reporter.
- Seal the plate with an Optical seal
- Open the plate reader software to create a read procedure. Set temperature to 37°C
- Select "Kinetic" run reading with a total read time of 30 min, and data collection intervals at 2.5 mins
- Save experiment in a designated place with an appropriate unique name
- When plate loader extdends, load plate. Ensure plate is loaded in correct orientation. Read the data.

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