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Fluorescent-Reporter Based Assay

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Protocol status: In development

We are still developing and optimizing this protocol

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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.


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


MATERIALS

 QuickExtract™ RNA Extraction Kit **Lucigen Catalog #QER090150**

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


STEP MATERIALS


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Protocol materials


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Troubleshooting

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 **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) **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)

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







- 3
 - 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


- 4
 - 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	$1.2 \mu\text{L} \times (N+1)$
	3 μM crRNA (N or E or RP)	0.8 μL	$0.8 \mu\text{L} \times (N+1)$
	3 μM IbCas12a	0.4 μL	$0.4 \mu\text{L} \times (N+1)$
	RNAse-Free Water	9.6 μL	$9.6 \mu\text{L} \times (N+1)$
	Total Volume	12 μL	$12 \mu\text{L} \times (N+1)$

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 fluorescence reporter as per Table 4

	Reagent Name	Volume per Reaction	Volume Total
	FAM-FQ	0.2 μL	$0.2 \mu\text{L} \times (N^* + 1)$
	RNAse-Free Water	25.8 μL	$25.8 \mu\text{L} \times (N^* + 1)$
	Total Volume	12 μL	$12 \mu\text{L} \times$



			(N* +1)
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Table 4: Fluorescence 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

- 5
 - Add 26 μ L 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 μ L of the RT-LAMP product from step 3 to each well containing the fluorescent reporter.
 - Add 12 μ L 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 extends, load plate. Ensure plate is loaded in correct orientation. Read the data.