Luna® SARS-CoV-2 RT-qPCR Multiplex Assay Kit

NEB #E3019S/L

96/480 reactions

Version 1.0_11/20

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Kit Components

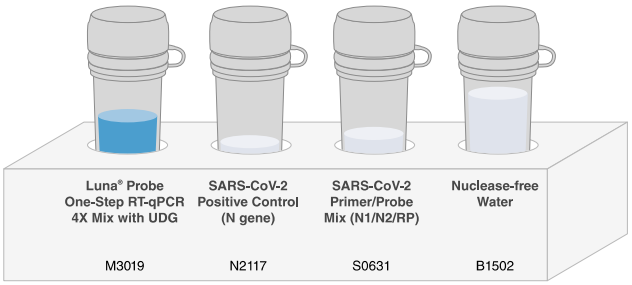
Reagents are shipped on ice and should be placed at -20°C upon receipt. All components will freeze at -20°C and must be thawed before use. Thaw components at room temperature and then place on ice or at 4°C during use. Store materials at -20°C after use. RT-qPCR Primer/Probe mix and Positive Control should be gently vortexed before reaction setup and Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M03019) should be mixed well by inverting.

Luna Probe One-Step RT-qPCR 4X Mix with UDG (4X)

SARS-CoV-2 Positive Control (N gene)*

SARS-CoV-2 Primer/Probe Mix (N1/N2/RP) (10X)

Nuclease-free Water



* *SARS-CoV-2 Positive Control is plasmid DNA containing the SARS-CoV-2 N gene and should not be used as an extraction control.*

Required Equipment/Materials Not Included

Disposable powder-free gloves and any additional PPE required

P2/P10, P200, and P1000 aerosol barrier tips

Sterile, nuclease-free 1.5 ml microcentrifuge tubes

Sterile, nuclease-free 2.0 ml, 5.0 ml, or 15 ml tubes

96-well, or 384-well PCR reaction plates, or 0.2 ml PCR reaction tube strips with separated tubes and lids (e.g., VWR 20170-004) or attached caps (e.g., VWR 20170-010)

Racks for 1.5 ml microcentrifuge tubes and 96-well 0.2 ml PCR reaction tubes

Cooler rack for 1.5 microcentrifuge tubes and 96-well 0.2 ml PCR reaction tubes

Acceptable surface decontaminants, for example: 10% bleach (1:10 dilution of commercial 5.25-6.0% sodium hypochlorite)

Laboratory marking pen

Appropriate disposal containers

Micropipettes (2 or 10 µl, 200 µl and 1,000 µl), Multichannel Micropipettes (5-50 µl)

-20°C Freezer (frost-free or nonfrost), 4°C Refrigerator

Thermocycler

PCR Work Station [UV lamp; Laminar flow (Class 100 HEPA filtered)], Vortex Mixer, Tabletop Microcentrifuge

Warnings and Precautions

- Follow standard guidelines to reduce sample and control reaction cross contamination
- Care should be taken when opening and using the N-gene control to prevent possible cross contamination
- **DO NOT** open assay tubes following RT-qPCR. Record assay results and dispose of assays immediately upon completion
- Waste should be disposed of in compliance with local, state and federal regulations
- Always use pipette tips containing aerosol barriers that are sterile and free of DNases and RNases
- Appropriate safety procedures should be followed at all times
- Reagents must be stored at -20°C when not in use

Introduction

The Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit is optimized for real-time qualitative detection of SARS-CoV-2 nucleic acid using hydrolysis probes. In a single tube, RNA is first converted to cDNA by a reverse transcriptase, then a DNA-dependent DNA polymerase amplifies the cDNA, enabling quantitation via real-time or quantitative PCR (qPCR). Probe-based qPCR/RT-qPCR monitors an increase in fluorescence upon 5´→ 3´ exonuclease cleavage of a quenched, target-specific probe to measure DNA amplification at each cycle of a PCR. At a point where the fluorescence signal is confidently detected over the background fluorescence, a quantification cycle or Cq value can be determined. Cq values can be used to evaluate relative target abundance between two or more samples.

In the Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit, Hot Start *Taq* DNA Polymerase is combined with a novel WarmStart®-activated reverse transcriptase, allowing dual control of enzyme activity via reversible, aptamer-based inhibition. This temperature-dependent activation helps to prevent undesirable non-specific priming and extension prior to thermocycling, providing added security for setting up reactions at room temperature. The engineered WarmStart Luna Reverse Transcriptase also possesses higher thermostability than many other RTs, allowing an optimal reaction temperature of 55°C.

The Luna Probe One-Step RT-qPCR Master Mix is supplied at 4X concentration and contains all the necessary components for One-Step RT-qPCR. It is formulated with a unique passive reference dye that is compatible across a variety of instrument platforms, including those that require a high or low ROX reference signal. The Reaction Mix also features dUTP/UDG for carryover prevention and a non-fluorescent visible dye for monitoring reaction setup. This visible dye does not overlap spectrally with fluorophores commonly used in qPCR and does not interfere with real-time detection.

The SARS-CoV-2 Primer/Probe mix provided with this kit contains primers and probes specific to two regions of the SARS-CoV-2 virus N-gene [based on sequences provided by the Centers for Disease Control and Prevention (CDC)]. However, they have been modified to contain different fluorophores (N1, HEX; N2, FAM) to enable simultaneous observation on two different channels of a real-time instrument. To ensure the integrity of the input material and absence of inhibition, an internal control (IC) primer and probe set, designed to amplify the human RNaseP gene, is also provided in the primer mix. The reverse primer of this target has been modified from the CDC design to target an exon/exon boundary to reduce background amplification from possible contaminating genomic DNA. Amplification of the IC is observed in the Cy5 channel. A positive control (PC) template (SARS-CoV-2 N-gene cloned into a plasmid) is also provided.

Sample Compatibility

- This kit has been evaluated using purified total RNA or total nucleic acid eluted in nuclease-free water or common elution buffers
- For purified nucleic acid eluted in water or AVE buffer (RNase-free water with 0.04% Sodium azide), input volumes can represent up to 50% of the reaction volume
- Material stored in TE or similar elution buffer should be kept to 5 µl (25% v/v) or less of the final reaction volume
- Material stored in PBS buffer should be kept to 2 µl (10% v/v) or less of the final reaction buffer
- Additional types of input may also be tolerated but % volume limits will need to be determined empirically

Luna SARS-CoV-2 RT-qPCR Multiplex Assay Protocol

1. Thaw Luna Probe One-Step RT-qPCR 4X Mix with UDG, SARS-CoV-2 Primer Mix (10X), Positive Control and Nuclease-free Water at room temperature.

2. Once completely thawed, mix each component by inversion, pipetting or gentle vortexing. Centrifuge briefly to collect liquid to the bottom of the tube and then place in a cold rack at 4°C or ice.

3. Detemine the total number of reactions per assay run. Each assay run should include the following:
    a) One Positive Control using the SARS-CoV-2 Positive Control provided in the kit, as template
    b) One Negative Control (NC) using Nuclease-free Water provided in the kit, as template
    c) t number of Test Samples

    The total number of reactions *n* = 1 (Positive Control) + 1 (Negative Control) + t (Test Samples)

    An overview of the reaction setup for each reaction type is described in the table below, assuming 20 µl reaction volumes as recommended for PCR tubes or 96-well plates. For 384-well plates, reaction volumes can be reduced to 5-10 µl.

	VOLUME PER 20 µl REACTION		
COMPONENT	TEST SAMPLES	POSITIVE CONTROL	NEGATIVE CONTROL
Luna Probe One-Step RT-qPCR 4X Mix with UDG	5 µl	5 µl	5 µl
SARS-CoV-2 Primer/Probe Mix (N1/N2/RP) (10X)	2 µl	2 µl	2 µl
Test Sample	2–10 µl	–	–
SARS-CoV-2 Positive Control (N gene)	–	2 µl	–
Nuclease-free Water	to 20 µl	11 µl	13 µl

4. Combine the components indicated below on ice for the number of reactions (*n*) being evaluated. Volumes include 10% overage to accommodate transfer loss from pipetting. The recommended input volume (v) is 2-10 µl for Test Samples and 2 µl for the Positive Control (as indicated in step 6 below, water will be used to make up any difference in volume between the PC and the Test Samples).

    SARS-CoV-2 RT-qPCR Assay Mix:

COMPONENT	VOLUME FOR ONE REACTION	VOLUME FOR *n* REACTIONS
Luna Probe One-Step RT-qPCR Mix with UDG	5 µl	5 µl x *n* x 1.1
SARS-CoV-2 Primer/Probe Mix (N1/N2/RP) (10X)	2 µl	2 µl x *n* x 1.1
Nuclease-free Water	(13-v) µl	(13-v) µl x *n* x 1.1

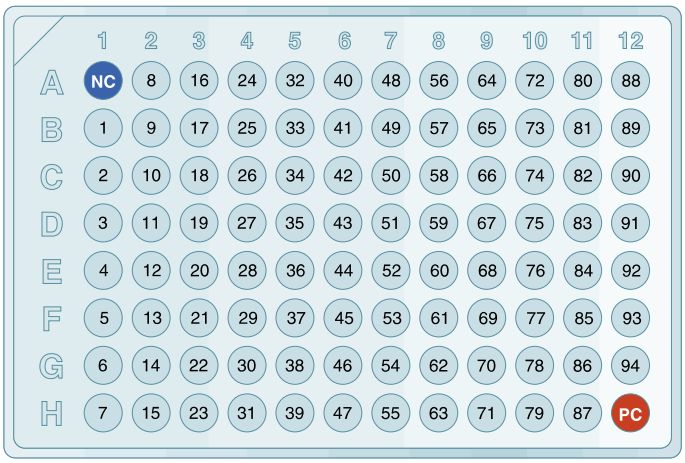
5. Mix thoroughly but gently by pipetting or vortexing, centrifuge briefly to collect liquid to the bottom of the tube.

6. Aliquot assay mix (20-v) µl into qPCR tubes or wells. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles. **If the input volume for the Test Sample is greater than 2 µl, add (v -2) µl nuclease-free water to the Positive Control tube or well.**

    For example: If *n* = 50 and the Test Sample volume is 5 µl, add 15 µl of the assay mix into 50 tubes or wells. Then add an additional 3 µl of water into the Positive Control tube or well.

7. Add v µl Test Sample, v µl nuclease-free water as Negative Control, and 2 µl Positive Control, to the appropriate qPCR tubes or wells on the plate. Mix thoroughly but gently by pipetting up and down, taking care to avoid bubbles.

   Example sample layout for a 96-well plate:



8. Seal tubes with flat, optically transparent caps; or seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.

9. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm).

10. Program real-time instrument with indicated thermocycling protocol (see table below). Ensure a plate read is included at the end of the extension step.

Programming the Real-time PCR Instrument

Use the scanning mode that will detect signal in all channels on the real-time instrument.

For faster results, the "Fast" ramp speed mode can be used where available (e.g., QuantStudio®, 7500 Fast instruments).

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Carryover Prevention	25°C	30 seconds	1
Reverse Transcription	55°C	10 minutes	
Initial Denaturation	95°C	1 minute	
Denaturation	95°C	10 seconds	45
Extension	60°C	30 seconds (+ plate read)	

Target fluorophore assignment:

TARGET	FLUOROPHORE/CHANNEL
2019-nCoV_N1	HEX
2019-nCoV_N2	FAM
Internal Control (RNase P)	Cy5

Data Analysis

1. For basic information regarding data analysis on specific real-time PCR instruments, please refer to the user manual of the respective instrument.

2. After the run is complete, inspect the amplification plot to ensure that the baseline threshold was set within the PCR exponential phase and above any background signal.

Usage Notes

Reaction Setup

Due to the dual hot-start feature of Luna One-Step Kits, it is not necessary to set up reactions on ice or preheat the thermocycler prior to use. For 96-well plates, a final reaction volume of 20 µl is recommended. For 384-well plates, a final reaction volume of 5-10 µl is recommended. When programming instrument cycling conditions, ensure a plate read is included at the end of the extension step.

Instrument Compatibility and ROX Normalization

This kit relies upon the detection of FAM, HEX and Cy5 flourescent signals. Please ensure that your instrument includes the ability to detect these fluorophores. Note that some instruments may require calibration prior to use.

In addition, the mix is formulated to include a universal ROX reference dye that is compatible with a variety of qPCR instrument types, including those that use no passive reference normalization and those that use a low or high concentration of passive reference dye (ROX). Therefore, no additional components are required to ensure compatibility with these instruments.

Interpretation of Results

SAMPLE	2019-nCoV_N1 (HEX)	2019-nCoV_N2 (FAM)	INTERNAL CONTROL (CY5)	INTERPRETATION
Positive Control	Cq < 27	Cq < 27	ND*	QC passed
Negative Control	ND	ND	ND	QC passed
Test Sample	ND	ND	ND	Invalid Test (Internal Control Failure)
	Cq < 40	ND	Cq < 40	Inconclusive – Retest
	ND	Cq < 40	Cq < 40	Inconclusive – Retest
	Cq < 40	Cq < 40	Cq < 35 or ND**	Positive
	ND	ND	Cq < 35	Negative

*** ND: not detected or detected with Cq > 40.**

****** When a test sample contains viral RNA yet only a few or no somatic cells, it is possible that the sample produces positive N1 and N2 amplification signal (Cq < 40) and negative RNase P signal (Cq > 35). This sample should be considered as Positive.

SARS-CoV-2 RT-qPCR Primer/Probe Sequences (5´→ 3´)

2019-nCoV_N1 Primer/Probe Set	**Sequence**
2019-nCoV_N1 Forward Primer	5´ GAC CCC AAA ATC AGC GAA AT 3´
2019-nCoV_N1 Reverse Primer	5´ TCT GGT TAC TGC CAG TTG AAT CTG 3´
2019-nCoV_N1 Probe	5´ HEX-ACC CCG CAT TAC GTT TGG TGG ACC-Q 3´

2019-nCoV_N2 Primer/Probe Set	**Sequence**
2019-nCoV_N2 Forward Primer	5´ TTA CAA ACA TTG GCC GCA AA 3´
2019-nCoV_N2 Reverse Primer	5´ GCG CGA CAT TCC GAA GAA 3´
2019-nCoV_N2 Probe	5´ 6-FAM-ACA ATT TGC CCC CAG CGC TTC AG-Q 3´

RNase P Primer/Probe Set	**Sequence**
RNase P Forward Primer	5´ AGA TTT GGA CCT GCG AGC G 3´
RNase P Reverse Primer	5´ CAA CTG AAT AGC CAA GGT GAG C 3´
RNase P Probe	5´ Cy5-TTC TGA CCT GAA GGC TCT GCG CG-Q 3´

Note: Q= quencher

Ordering Information

NEB #	PRODUCT	SIZE
E3019S/L	Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit	96/480 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
M3019S/L	Luna Probe One-Step RT-qPCR 4X Mix with UDG	100/500 reactions
M3019X/E	Luna Probe One-Step RT-qPCR 4X Mix with UDG	1,000/2,000 reactions
T2010S	Monarch® Total RNA Miniprep Kit	50 preps
E3032S	Luna Cell Ready Lysis Module	100 reactions

Revision History

REVISION #	DESCRIPTION	DATE
1.0		11/20

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