INSTRUCTION MANUAL

Luna® Cell Ready Probe One-Step RT-qPCR Kit

NEB #E3031S

Version 2.0_2/24

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Kit Components

This kit contains two components, the Luna Cell Ready Lysis Module (NEB #E3032S) and Luna Universal Probe One-Step RT-qPCR Kit (NEB #E3006L). Both should be stored at –20°C upon receipt and the RT-qPCR Kit should be protected from light. If desired, the Luna Cell Ready Lysis Buffer (2X) can be stored at 4°C during ongoing use. This kit has a shelf-life of 24 months when stored properly under these conditions.

Luna Cell Ready Lysis Module, NEB #E3032S, 100 reactions (50 µl)

Luna Cell Ready Lysis Buffer (2X)	2 x 1.4 ml
DNase I (RNase-free) (10X)	0.5 ml
Luna Cell Ready RNA Protection Reagent (25X)	0.25 ml
Luna Cell Ready Protease (25X)	0.2 ml
Luna Cell Ready Stop Solution (10X)	0.6 ml

Luna Universal Probe One-Step RT-qPCR Kit, NEB #E3006L, 500 reactions (20 µl)

Luna Universal Probe One-Step Reaction Mix (2X)	5 x 1 ml
Luna WarmStart® RT Enzyme Mix (20X)	1 x 0.5 ml
Nuclease-free Water	3 x 1.5 ml

Required Materials Not Included

- Phosphate-buffered saline (PBS), chilled
- Target-specific primers
- Cells
- Eppendorf tubes, PCR strip tubes, PCR plates
- qPCR instrument
- Pipettors and pipette tips (to minimize cross contamination, filter tips should be used)

Introduction

The Luna Cell Ready Probe One-Step RT-qPCR Kit consists of: 1) the Luna Cell Ready Lysis Module (NEB# E3032S) and 2) the Luna Universal Probe One-Step RT-qPCR Kit (NEB# E3006L). The Luna Cell Ready Probe One-Step RT-qPCR Kit provides all the necessary components for direct RNA detection and quantitation, bypassing the need for RNA extraction and purification.

Coordinating the actions of DNase I and the Luna Cell Ready Protease, the Luna Cell Ready Lysis Module offers a simple workflow resulting in effective cell lysis, RNA release, and genomic DNA removal simultaneously in a 15-minute protocol. The Lysis Module includes a unique Luna Cell Ready RNA Protection Reagent that maintains RNA integrity during cell lysis. The lysis capacity spans 10–100,000 cells in a 50 µl lysis reaction. Up to 2 µl lysate (equivalent to RNA from 0.2–4,000 cells) can be transferred into 20 µl downstream RT-qPCR reactions. In addition, the Luna Cell Ready Lysis Buffer contains a blue tracking dye, providing a visual indicator that can be followed throughout the entire reaction setup. This visible dye does not overlap spectrally with fluorophores or reference dyes commonly used in qPCR and does not interfere with real-time detection.

The Luna Universal Probe One-Step RT-qPCR Kit is compatible with probe-based real-time quantitation of target RNA sequences via multiple fluorescence channels of most real-time instruments. In the Luna Universal Probe One-Step RT-qPCR Kit, Hot Start *Taq* DNA Polymerase is combined with a novel Luna WarmStart Reverse Transcriptase, allowing dual control of enzyme activity via reversible, aptamer-based inhibition. The Luna Universal Probe One-Step RT-qPCR Kit can (in a singleplex or in a multiplex reaction) detect and quantitate RNA transcripts in the cell lysate prepared directly from the Luna Cell Ready Lysis Module.

The Luna Cell Ready Probe One-Step RT-qPCR Kit has been tested for robust and sensitive RNA detection from cultured mammalian cells, including adherent cells, suspension cells and cryopreserved cells. Please refer to the kit FAQs at www.neb.com/E3031 for the most up-to-date list of cell lines/cells tested.

For larger volume requirements, customized and bulk packaging is available through the NEB Customized Solutions department. Please contact custom@neb.com for more information.

General Tips and Considerations

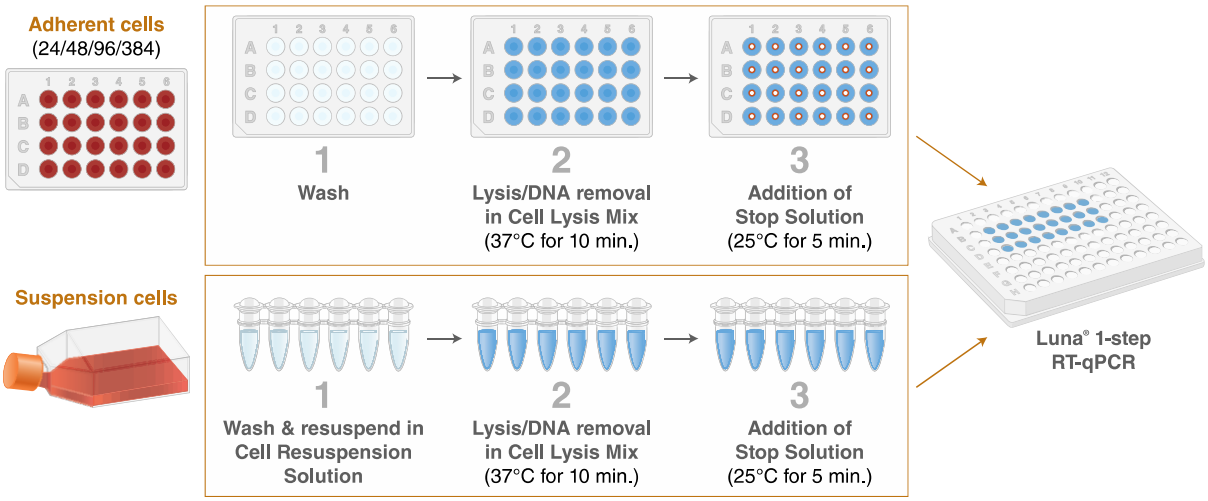
- General rules for cell culture should be followed.
- Intact RNA is essential for sensitive RT-qPCR detection. Apply precautions such as using filter tips, wearing gloves to avoid contamination, and minimizing sample handling time.
- To prevent undesirable bubbles from forming during cell lysis, use gentle pipetting and a brief spinning procedure. (Excess bubbles will prevent optimal cell lysis, causing low efficiency and inaccurate volume transfer during subsequent steps. After pipetting into the qPCR plate, if 1–2 small bubbles are present at the top of the well, the assay can proceed, as these bubbles will typically dissipate during the first denaturation step of the PCR.)
- The Luna Cell Ready Lysis Module has a lysis capacity of 10–100,000 cells in a 50 µl lysis reaction. Typically, 1–2 µl cell lysate (equivalent to RNA from 0.2–4,000 cells) can be transferred into a 20 µl downstream RT-qPCR. Most transcripts can be detected from 20 to 200 cells in a typical 20 µl reaction.
- The cell lysate can easily comprise up to 10% of the one-step RT-qPCR volume (e.g., 2 µl cell lysate in 20 µl RT-qPCR experiment).
- Cell lysates can be stored on ice up to five hours, at -20°C for up to five days and at -80°C for long term storage. Cell lysates are stable for up to five freeze and thaw procedures. Optimal results are seen using the lysates in downstream RT-qPCR experiments as soon as possible. Similarly, RT-qPCR reactions should proceed immediately after setup for best results.
- Some cell lines are difficult to lyse or may contain components inhibitory to RT-qPCR; therefore, several cell dilutions may be used to determine the appropriate range of cell numbers for lysis and RT-qPCR. To evaluate primers and targets for desirable linearity and efficiency, purified RNA controls should be used.
- During cell lysis, most genomic DNA is removed effectively. However, the use of RT-qPCR primers that span exons may further reduce qPCR signals from genomic DNA and therefore improve sensitivity.
- It is recommended to run all qPCR reactions in triplicate. This permits exclusion of outlier traces (e.g., due to unexpected plate issues, edge effects, or other problems) while maintaining accurate quantitation.
- When using multichannel pipettes, care should be taken to ensure consistency of pipetting volume.
- Primers purified with standard desalting methods are sufficient for use in Luna qPCR/RT-qPCR. In some cases, HPLC or PAGE purification may be helpful for assays that require increased sensitivity.

Luna Cell Ready Probe One-Step RT-qPCR Kit Protocol

Before Use

- *Prepare cells in advance (various cell types such as adherent, suspension, or cryopreserved can be used) and ensure cells are intact before lysis.*
- *Consider testing several cell dilutions to determine the appropriate input range to use for lysis and RT-qPCR. Although a standard curve is not required for high throughput screening experiments, it is still recommended.*
- *It is important to use only a monolayer of cells in well-plate formats. We recommend using Surface Areas and Guide for Recommended Medium Volumes from Corning® as a starting point for preparing cell cultures.*
- *Ensure that all components are thawed and mixed prior to use. Once thawed, place on ice prior to use. Workflow using Luna Cell Ready One-Step RT-qPCR Kit.*

Workflow using Luna Cell Ready One-Step RT-qPCR Kit.



Part I. Cell Lysate Preparation using Luna Cell Ready Lysis Module

Step 1. Processing Cells

Cell Resuspension Solution (CRS) is recommended for resuspending/diluting cells to reduce RNA damage during the handling process. When only a fraction of the whole sample is to be used (e.g., adherent cells grown in vessels other than 24/48/96/384 well plates, suspension cells, or cryopreserved cells), you should resuspend with CRS to protect your samples' RNA during processing. CRS is not required for adherent cells in 24/48/96/384 well plates.

Prepare Cell Resuspension Solution (CRS) by diluting Luna Cell Ready RNA Protection Reagent (25X) to 1X with cold PBS (e.g. for each sample, mix 2 µl Luna Cell Ready RNA Protection Reagent with 48 µl of 1X PBS to make 50 µl Cell Resuspension Solution).

<table>
<tr><th>Adherent cells in
24/48/96/384 well plates</th><th>Adherent cells
grown in other vessels</th><th>Suspension cells</th><th>Cryopreserved cells</th></tr>
<tr><td rowspan="2">1. Remove all cell culture media.
2. Rinse briefly with cold PBS and aspirate PBS*.
3. Store on ice and proceed to lysis within 10 mins.</td><td>Detach cells using common sub-culturing techniques.</td><td>—</td><td>Quickly thaw the cell stock at 37°C.</td></tr>
<tr><td colspan="3">1. Transfer the desirable volume of cells and spin down the cell pellets. Carefully remove the supernatant.
2. Rinse briefly with cold 1X PBS and aspirate PBS*.
3. Resuspend cells with CRS (up to 20,000 cells/µl). Store on ice and proceed to lysis within 10 mins.</td></tr>
</table>

* For high throughput screening (e.g., adherent cells in 96 wells or 384 well plates), cell wash is optional if medium removal can be completed via a plate flipping step.

Step 2. Prepare Cell Lysis Mix

1. Thaw Luna Cell Ready Lysis Buffer and Luna Cell Ready Stop Solution at room temperature, then place on ice with all other components. After thawing completely, briefly mix each component by inversion.
2. Prepare Cell Lysis Mix of all components, adding the Luna Cell Ready Protease immediately before use.
   Mix thoroughly by pipetting gently. Centrifuge briefly to collect the solution to the bottom of the tube and store on ice.
   For best results, the Cell Lysis Mix should be used immediately (within 15 minutes).

COMPONENT	40 µl CELL LYSIS MIX	FINAL CONCENTRATION
Luna Cell Ready Lysis Buffer (2X)	25 µl	1X
DNase I (RNase-free) (10X)	5 µl	1X
Luna Cell Ready RNA Protection Reagent (25X)	2 µl	1X
Luna Cell Ready Protease (25X)	2 µl	1X
Nuclease-free Water	6 µl	

Up to 2,000 cells per µl lysis reaction is recommended. In a typical 50 µl lysis reaction, 100,000 cells can be lysed.

Step 3. Cell Lysis

For lysis using cells resuspended from adherent cells grown in vessels other than 24/48/96/384 well plates, suspension cells, or cryopreserved cells: Mix up to 5 µl of cells resuspended in CRS with the Cell Lysis Mix to a final volume of 45 µl. Gently pipet up and down 6 times. Incubate the lysis reaction at 37°C for 10 min.*

For lysis of adherent cells in 24/48/96/384 well plates: Aliquot an appropriate volume of Cell Lysis Mix into each well as indicated in the following table and incubate the reaction at 37°C for 10 min.*

For most efficient lysis, automatic shaking is recommended for cell densities higher than 200 cells/µl.

CULTURE PLATE	CELL LYSIS MIX PER WELL
24 well	160 µl
48 well	80 µl
96 well	40 µl
384 well	8 µl

* Note: Lysis at room temperature is an option if cell density is less than 200 cells/µl in the lysate.

Step 4. Lysis Termination

Add 10X Luna Cell Ready Stop Solution to a final concentration of 1X and mix well by pipetting up and down 6 times. Centrifuge briefly to collect the solution to the bottom of the tube. Incubate at 25°C for 5 min.

For 96 well plates, we found that adding 5 µl of Luna Cell Ready Stop Solution (10X) to the 40 µl lysis solution (± 10% cells and residual wash buffer) was sufficient for complete lysis.

Cell lysates can be stored on ice for up to five hours, at -20°C for up to five days and at -80°C for long term storage. Cell lysate is stable for up to five freeze and thaw procedures.

Part II. RNA Detection using Luna Universal Probe One-Step RT-qPCR Kit

For best results, it is recommended to run each lysate sample and control in triplicate.

1. Determine the total volume for the appropriate number of reactions (adding 10% overage) and prepare an assay mix of all components except for cell lysate. Mix thoroughly by gentle pipetting or vortexing. Centrifuge briefly to collect the solution to the bottom of the tube.

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Luna Universal Probe One-Step Reaction Mix (2X)	10 µl	1X
Luna WarmStart RT Enzyme Mix (20X)	1 µl	1X
Forward Primer (10 µM)	0.8 µl	0.4 µM
Reverse Primer (10 µM)	0.8 µl	0.4 µM
Probe (10 µM)	0.4 µl	0.2 µM
Cell Lysate	variable	≤ 2 µl* (up to 4,000 cells)
Nuclease-free Water	to 20 µl	

* In general, 1 µl of cell lysate is recommended. However, lysate input may be optimized for best results. It is not recommended for the lysate to exceed more than 10% of the RT-qPCR reaction volume.

2. Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.
3. Add cell lysate to qPCR tubes or plate. Seal tubes with flat, optically transparent caps; seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.
4. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm). **RT-qPCR experiment should proceed immediately after setup for best results.**
5. Program real-time instrument with indicated thermocycling protocol (see table below). Ensure that a plate read is included at the end of the extension step.
6. Confirm selection of appropriate detection channel(s) for the fluorophore(s) present in the assay in the real-time instrument.
7. For faster results, the "Fast" ramp speed mode can be used where available (e.g., Applied Biosystems StepOnePlus®, QuantStudio®, 7500 Fast instruments).

CYCLE STEP	TEMP	TIME	CYCLES
Reverse Transcription	55°C*	10 minutes	1
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	10 seconds	40–45
Extension	60°C	30 seconds** (+ plate read)	

***A 55°C RT step temperature** is optimal for Luna WarmStart Reverse Transcriptase.
To insure best performance and full WarmStart activation avoid using a temperature of < 50°C.

* For Applied Biosystems real-time instruments use a 60 second extension step.

Usage Notes

Primer Design

The use of qPCR primer design software (e.g., Primer3) maximizes the likelihood of amplification success while minimizing nonspecific amplification and primer dimers. Targets with balanced GC/AT content (40–60%) tend to amplify most efficiently. Where possible, enter sufficient sequence around the area of interest to enable robust primer design and use search criteria that permits cross-referencing against relevant sequence databases (to avoid potential off-target amplification). It is advisable to design primers across known RNA splicing sites in order to prevent amplification from genomic DNA.

Primer Concentration

For most targets, a final concentration of 400 nM (each primer) will provide optimum performance. If needed, primer concentrations can be optimized between 100–900 nM. Probes should be included at 200 nM for best results. Probe concentration can be optimized in the range of 100–500 nM.

Multiplexing

When determining which fluorophores to include in a multiplex reaction, be sure to choose compatible reporter dyes and quenchers (e.g., those that can be accommodated by the chosen real-time instrument with minimal overlap in fluorescence spectra). For ROX-dependent instruments, avoid ROX-labeled probes. Include 400 nM of forward and reverse primers and 200 nM probe for each target to be detected in the reaction. For targets that differ significantly in abundance, use of a lower primer concentration (e.g., 200 nM) for the more abundant target(s) is recommended. Adjust concentrations if necessary based on performance (primer 100–900 nM, probe 100–500 nM). When loading a qPCR protocol onto the real-time instrument, be sure to select the appropriate optical channels, as some instruments have a single channel recording mode that would prevent multiplex data collection and analysis. The functionality of the primer and probe sets should be tested individually before attempting a multiplex reaction.

Amplicon Length

To ensure successful and consistent qPCR results, it is important to maximize PCR efficiency. An important aspect of this is the design of short PCR amplicons (typically 70–200 bp). Some optimization may be required for targets that exceed that range.

Template Preparation and Concentration

The Luna Cell Ready Lysis Module has a lysis capacity of 10–100,000 cells in a 50 µl lysis reaction. Typically, 1–2 µl cell lysate (equivalent to RNA from 0.2–4,000 cells) can be transferred into a 20 µl downstream RT-qPCR). Most transcripts can be detected from 20 to 200 cells in a typical 20 µl reaction.

ROX Reference Dye

Some real-time instruments recommend the use of a passive reference dye (typically ROX) to overcome well-to-well variations that could be caused by bubbles, small differences in volume, and autofluorescence from dust or particulates in the reaction. Luna mixes are formulated with a universal 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.

Carryover Contamination Prevention

RT-qPCR is an extremely sensitive method, and contamination in new RT-qPCR assays with products from previous amplification reactions can cause a variety of issues, such as false positive results and a decrease in sensitivity. The best way to prevent this "carryover" contamination is to practice good laboratory procedures and avoid opening the reaction vessel post amplification. However, to accommodate situations where additional anti-contamination measures are desired, Luna qPCR mixes contains a mixture of dUTP/ dTTP that results in the incorporation of dU into the DNA product during amplification. Pretreatment of qPCR/RT-qPCR experiments with uracil DNA glycosylase (UDG) will eliminate previously-amplified uracil-containing products by excising the uracil base to produce a non-amplifiable DNA product.

To enable carryover prevention, 0.025 units/µl Antarctic Thermolabile UDG (NEB #M0372) should be added to the reaction mix. To maximize elimination of contaminating products, set up the qPCR/RT-qPCR experiments at room temperature or include a 10 min incubation step at 25°C before the initial denaturation step. The use of a thermolabile UDG is important, as complete inactivation of the UDG is required to prevent destruction of newly synthesized qPCR products.

Reaction Setup and Cycling Conditions

Due to the dual hot-start feature of the Luna Universal One-Step RT-qPCR Kit, 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 10 µl is recommended.

When programming instrument cycling conditions, ensure a plate read is included at the end of the extension step, and a denaturation (melt) curve after cycling is complete to analyze product specificity.

Amplification for 40 cycles is sufficient for most applications, but for very low input samples, 45 cycles may be used.

Troubleshooting Guide

OBSERVATION	POSSIBLE CAUSE(S)	SOLUTION(S)
Delayed qPCR traces or no amplification	Too many cells were used in the lysis reaction	• In general, up to 100,000 cells can be lysed successfully per 50 µl lysis reaction. If more cells are used, scale up the lysis reaction accordingly.
	Too few cells were used in the lysis reaction	• Increase cell numbers up to 2,000 per µl lysate for rare transcripts
	Excess amount of culture medium or PBS remained (e.g., during high throughput screening)	• Remove medium/PBS as thoroughly as possible by good aspiration or plate-flip techniques. PBS carryover should be ≤ 10% of total lysis reaction volume.
	Components in the lysis reaction are not fully inactivated by stop solution or excessive stop solution was used	• Add stop solution to the lysis reaction and mix well • Use the recommended volume of Luna Cell Ready Stop Solution
	Some cell lines may contain high levels of inhibitors for cell lysis or RT-qPCR	• The optimal input cell number for lysis may differ for different cell lines or culture conditions. Please refer to the kit FAQs at www.neb.com/E3031 for the most up-to-date list of cell lines/cells tested. • Try to reduce cell input up to 100-fold
	RNA was degraded during harvest, wash or cell lysis	• Ensure cells are intact prior to lysis • Include Luna Cell Ready RNA Protection Reagent for cell resuspension and dilution • Proceed to RT-qPCR immediately after lysis
	RNA was degraded during lysate storage	• In general, cell lysates should be kept on ice no more than five hours or at -20°C for up to five days. For longer storage, -80°C is recommended. • Lysates can typically tolerate up to five freeze and thaw cycles • For best result, cell lysates containing < 2 cells/µl should be used immediately
	Cell lysis is not efficient, RNA is not fully released	• Use recommended amount of Luna Cell Ready Protease • Use 37°C for cell lysis. Lysis time can be extended up to 20 minutes, if needed • Reduce cell input to 100-fold
	The sample does not contain the target RNA	• Verify RT-qPCR detection using purified RNA • If positive RNA control is available, this can be spiked into the cell lysate to confirm detection
	Incorrect channel selected on the real-time instrument	• Verify correct instrument optical settings

Troubleshooting Guide (Cont'd)

OBSERVATION	POSSIBLE CAUSE(S)	SOLUTION(S)
Standard curve using cell dilutions has a poor correlation coefficient or undesirable efficiency (outside of 90%–110%)	Reaction conditions are incorrect or cycling protocol is incorrect	• Verify that all steps of the protocol were followed correctly • Refer to the proper RT-qPCR cycling protocol in this user manual. Use a 55°C RT step temperature. For ABI instruments, use a 1 minute for 60°C annealing/extension step.
	Inaccurate pipettes or inaccurate serial dilutions of cells	• Ensure pipettes are calibrated regularly and use proper pipetting techniques • Cell resuspension is heterogeneous; mix well before pipetting
	High level of inhibitors from cellular components cause efficiency above 110%	• Reduce the input cell number up to 100-fold • Shaking plates during in-well lysis may lead to more effective lysis
	RNA was degraded during cell lysis or RT-qPCR setup	• Avoid exposing lysates to room temperature after lysis • Assay lysate as soon as possible • RT-qPCR should proceed immediately after setup for best results. (Store at 4°C for no more than 5 hours).
	Threshold is improperly set for the qPCR traces	• Verify the threshold is set in the exponential region of qPCR traces
Inconsistent qPCR traces for triplicate data	Improper pipetting during RT-qPCR assay set-up	• Cell lysates contain detergents; pay attention to ensure accurate pipetting (e.g., no leftover in the pipette tip)
	qPCR plate film has lost its seal, causing evaporation and different fluorescence values	• Ensure the qPCR plate is properly sealed • Exclude problematic trace(s) from data analysis
	Poor mixing of reagents during RT-qPCR set-up or bubbles cause an abnormal qPCR trace	• Ensure thorough mixing of reagents after thawing • Centrifuge the qPCR plate after setup • Exclude outlier trace(s) from data analysis
Lower fluorescence signal or low efficiency for certain genes in a multiplex assay compared to in a singleplex assay	Reaction components are consumed by the amplification of abundant genes. Low abundant genes have limited components available for amplification.	• For targets that differ significantly in abundance, use of a lower primer concentration (e.g., 200 nM) for the more abundant target(s) is recommended. Adjust concentrations if necessary based on performance (primer 100–900 nM, probe 100–500 nM).
Signal in the No-RT control	Incomplete genomic DNA digestion	• Use the recommended amount of DNase I and Luna Cell Ready Protease • Mix lysis reaction during 37°C incubation • Reduce cell input up to 100-fold
	Cross contamination from RT-qPCR products	• Avoid opening RT-qPCR reactions • Perform UDG treatment
Further questions related to RT-qPCR		• Refer to the troubleshooting and FAQ's sections of Luna Universal One-Step RT-qPCR kit at www.neb.com/E3006

Ordering Information

NEB #	PRODUCT	SIZE
E3031S	Luna Cell Ready Probe One-Step RT-qPCR Kit	1 kit
COMPANION PRODUCTS		
E3032S	Luna Cell Ready Lysis Module	100 reactions
E3006S/L	Luna Universal Probe One-Step RT-qPCR Kit	200/500 reactions
E3006X	Luna Universal Probe One-Step RT-qPCR Kit	1,000 reactions
E3006E	Luna Universal Probe One-Step RT-qPCR Kit	2,500 reactions

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