INSTRUCTION MANUAL

Luna® Universal One-Step RT-qPCR Kit

NEB #E3005S/L/X/E

200/500/1,000/2,500 reactions

Version 4.0_10/20

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Kit Components

This product should be stored at –20°C, protected from light, and has a shelf-life of 24 months when stored properly under these conditions. The Reaction Mix is stable for at least 30 freeze/thaw cycles, and for short-term storage may be stored at 4°C, protected from light, for up to 1 month. The RT Enzyme Mix should be stored at –20°C.

All components are provided in volumes sufficient for preparation of up to 200 reactions (NEB #E3005S), 500 reactions (NEB #E3005L), 1,000 reactions (NEB #E3005X) or 2,500 reactions (NEB #E3005E). The Reaction Mix is supplied in 1 ml aliquots and Nuclease-free Water is supplied in 1.5 ml aliquots for NEB #E3005 S,L,X and for NEB #E3005E the Reaction Mix and Nuclease-free Water are supplied in 25 ml bottles.

Luna Universal One-Step Reaction Mix (2X)

Luna WarmStart® RT Enzyme Mix (20X)

Nuclease-free Water

Required Materials Not Included

Target-specific primers

RNA template

PCR strip tubes or microcentrifuge tubes (for reaction setup)

qPCR tubes or qPCR plates and seals

qPCR instrument

Introduction:

The NEB Luna Universal One-Step RT-qPCR Kit is optimized for dye-based real-time quantitation of target RNA sequences via the SYBR®/FAM fluorescence channel of most real-time instruments. Dye-based qPCR/RT-qPCR uses real-time fluorescence of a double-stranded DNA (dsDNA) binding dye, most commonly SYBR Green I, to measure DNA amplification after each PCR cycle. At a point where the fluorescence signal is confidently detected over the background fluorescence, a quantification cycle or C_q value can be determined. C_q values can be used to evaluate relative target abundance between two or more samples, or to calculate absolute target quantities in reference to an appropriate standard curve derived from a series of known dilutions.

One-Step RT-qPCR provides a convenient and powerful method for RNA detection and quantitation. In a single tube, RNA is first converted to cDNA by a reverse transcriptase, and then a DNA-dependent DNA polymerase amplifies the cDNA, enabling quantitation via qPCR.

In the Luna One-Step RT-qPCR 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**. For difficult targets/templates, higher RT step temperatures of **up to 60°C** can be used without compromising Luna performance.

Note that to ensure full activation of the WarmStart Luna RT, incubation at temperatures lower than 50°C is not recommended.

The Luna Universal One-Step Reaction Mix is supplied at 2X concentration and contains Hot-Start Taq DNA Polymerase, dNTPs, a fluorescent dsDNA-binding dye, and all required buffer components. 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 for carryover prevention and a non-fluorescent visible dye for monitoring reaction setup. This visible dye does not overlap spectrally with the included dsDNA-binding dye and does not interfere with real-time detection.

The Luna WarmStart RT Enzyme Mix is supplied at 20X concentration and contains Luna WarmStart Reverse Transcriptase as well as Murine RNase Inhibitor to aid in preventing RNA degradation (see also template preparation in Usage Notes). It is compatible with various RNA sample types (total RNA, poly(A)-RNA, etc.) and sources.

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

General Tips and Considerations

- RT-qPCR is a sensitive RNA detection method. Proper sterile technique and careful pipetting should be used to avoid contamination and ensure accurate quantitation results.
- Ensure that all components are thawed and mixed prior to use. Once thawed, place on ice prior to use.
- We recommend running all 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 pipetting into the qPCR plate, it is advisable to avoid the formation of bubbles. If 1–2 small bubbles are present at the top of the liquid after loading, the assay can proceed, as these bubbles will typically resolve during the first denaturation step of the PCR.
- The “SYBR® Green” or “SYBR/FAM” channel of the real-time instrument should be used for the Luna Universal One-Step RT-qPCR Kit. On some instruments, selecting a single channel for data collection can result in faster experiment times.
- A denaturation or melt curve step should be added at the end of the RT-qPCR cycling protocol to evaluate amplification specificity.
- When using multichannel pipettes, care should be taken to ensure consistency of pipetting volume.
- Primers purified with standard desalting are sufficient for use in Luna qPCR/RT-qPCR. HPLC or PAGE purification may be helpful for assays that require increased sensitivity.

Luna Universal One-Step RT-qPCR Kit Protocols

Before Use

- *Prepare RNA of interest using desired RNA extraction and purification methods. Determine concentration by OD_{260} absorbance.*
- *Make dilutions of RNA to be used for the standard curve. These should be prepared fresh before each experiment and can be diluted in either water or TE.*

Reaction Setup: *For best results, we recommend running each RNA standard and sample in triplicate.*

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Luna Universal 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
Template RNA	variable	< 1 µg (total RNA)*
Nuclease-free Water	to 20 µl	

*See Usage Notes for additional guidelines on primer design and template preparation/concentration.

1. Thaw Luna Universal One-Step Reaction Mix and other reaction components at room temperature, then place on ice. After thawing completely, briefly mix each component by inversion, pipetting or gentle vortexing.
2. Determine the total volume for the appropriate number of reactions, adding 10% overage, and prepare assay mix of all components except RNA template accordingly. Mix thoroughly but gently by pipetting or vortexing. Collect liquid to the bottom of the tube by brief centrifugation.
3. Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.
4. Add RNA template 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.
5. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm).
6. 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.

Use the SYBR or SYBR/FAM scan mode setting on the real-time instrument.

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)	
Melt Curve	60-95°C***	various	1

* **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.

*** Follow real-time instrument recommendations for melt curve step.

Data Analysis and Expected Results

Analyze data according to real-time instrument manufacturer instructions.

Please refer to the MIQE guidelines [Bustin, S.A., Benes, V., et al. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem., 55(4): 611−622.] for a full discussion of qPCR data analysis appropriate for peer-reviewed publication.

Briefly, this includes the following steps:

1. Determine the efficiency of the standard curve by plotting the log of the input concentration against the C_q. This can be done automatically in most qPCR instrument software packages or via the NEB online qPCR tool (accessible through NEBiocalculator® at NEBiocalculator.neb.com). The linear fit of this data should have a slope of -3.6 to -3.1, corresponding to a reaction efficiency of 90–110%.
2. Verify that the linear fit of the standard curve data has a correlation coefficient (R^2) value of ≥ 0.98.
3. Determine the reaction specificity by evaluating the melt profile. Amplification of the target sequence should typically result in the observation of a single melting peak. The melt profiles of the negative (no template) control samples should also be evaluated. The presence of NTC profiles that overlap the positive sample profiles may indicate the presence of contamination. Any samples whose melt profiles overlap the profile of the negative (no template) control samples should not be evaluated.
4. Evaluate any unknown samples with respect to appropriate standard curves or control samples, taking any dilution factors into account.

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 permit robust primer design and use search criteria that permit cross-reference 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.

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

Luna RT-qPCR is compatible with RNA samples prepared through typical nucleic acid purification methods. Prepared RNA should be stored in an EDTA-containing buffer (e.g., 1X TE) for long-term stability, and dilutions should be freshly prepared for a qPCR experiment in either TE or water. Note that the quality of RNA templates can greatly affect RT-qPCR efficiency. RNA should be handled with appropriate precautions to prevent RNase or DNase contamination. Use of nuclease-free water (provided) is strongly recommended. Where useful, RNA may be treated with DNase I to remove contaminating genomic DNA.

Generally, a useful concentration of standard and unknown material will be in the range of 10^8 copies to 10 copies. Note that for dilutions in the single-copy range, some samples will contain multiple copies and some will have none, as defined by the Poisson distribution. For total RNA, Luna One-Step Kits can provide linear quantitation over an 8-order input range of 1 µg – 0.1 pg. For most targets, a standard input range of 100 ng – 10 pg total RNA is recommended. For purified mRNA, input of ≤ 100 ng is recommended. For in vitro-transcribed RNA, input of □ 10^9 copies is recommended.

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. The use of a thermolabile UDG is important, as complete inactivation of the UDG is required to prevent destruction of newly synthesized qPCR products.

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 minute incubation step at 25°C before the initial denaturation step.

Reaction Setup and Cycling Conditions

Due to 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 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

Note: For additional assistance please refer to product FAQ's at www.neb.com/E3005.

PROBLEM	POSSIBLE CAUSE	SOLUTION(S)
qPCR traces show low or no amplification	Incorrect RT step temperature or RT step omitted	• For typical use, a **55°C** RT step temperature is optimal for the Luna WarmStart Reverse Transcriptase (see cycling protocol)
	Cycling protocol is otherwise incorrect	• Refer to the proper RT-qPCR cycling protocol in this user manual
	Reagent omitted from RT-qPCR assay Reagent added improperly to RT-qPCR assay	• Verify all steps of the protocol were followed correctly
	Incorrect reporter dye selected for the qPCR thermal cycler	• Select FAM/SYBR on the qPCR instrument
	RNA template or reagents are contaminated or degraded	• Prepare high quality RNA without RNAse/DNase contamination • Confirm template input amount • Confirm the expiration dates of the kit reagents • Verify proper storage conditions provided in this user manual • Rerun the RT-qPCR assay with fresh reagents
Inconsistent qPCR traces for triplicate data	Improper pipetting during RT-qPCR assay set-up	• Ensure proper pipetting techniques
	qPCR plate film has lost its seal, causing evaporation in the well. The resulting qPCR trace may show significantly different fluorescence values relative to its replicates.	• Ensure the qPCR plate is properly sealed before inserting into the qPCR thermal cycler. • Exclude problematic trace(s) from data analysis
	Poor mixing of reagents during RT-qPCR set-up	• Make sure all reagents are properly mixed after thawing them
	Bubbles cause an abnormal qPCR trace	• Avoid bubbles in the qPCR plate • Centrifuge the qPCR plate prior to running it in the thermal cycler • Exclude problematic trace(s) from data analysis

PROBLEM	POSSIBLE CAUSE	SOLUTION(S)
Standard curve has a poor correlation coefficient/ efficiency of the standard curve falls outside the 90-110% range	Cycling protocol is incorrect	• 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 60°C annealing/extension step
	Presence of outlying qPCR traces	• Omni data produced by qPCR traces that are clearly outliers caused by bubbles, plate sealing issues, or other experimental problems
	Improper pipetting during RT-qPCR assay set-up	• Ensure that proper pipetting techniques are used
	Reaction conditions are incorrect	• Verify that all steps of the protocol were followed
	Bubbles cause an abnormal qPCR trace	• Avoid bubbles in the qPCR plate • Centrifuge the qPCR plate prior to running it in the thermal cycler
	Poor mixing of reagents	• After thawing, make sure all reagents are properly mixed
	Threshold is improperly set for the qPCR traces	• Ensure the threshold is set in the exponential region of qPCR traces • Refer to the real-time instrument user manual to manually set an appropriate threshold
Melt curve shows different peaks for low input samples	Non-template amplification is occurring Infrequently, denaturation of a single species can occur in a biphasic manner, resulting in two peaks	• Compare melt curve of NTC to samples • Redesign primers with a Tm of 60°C or use our Tm calculator to determine the optimal annealing temperature of the primers • Perform a primer matrix analysis to determine optimal primer concentrations
No template control qPCR trace shows amplification/NTC C_q is close to or overlapping lower copy standards	Reagents are contaminated with carried-over products of previous qPCR (*Melt curve of NTC matches melt curve of higher input standards*)	• Replace all stocks and reagents • Clean equipment and setup area with a 10% chlorine bleach • Consider use of 0.025 U/µl Antarctic Thermolabile UDG to eliminate carryover products
	Primers produce non-specific amplification (*Melt curve of NTC matches melt curve of higher input standards*)	• Redesign primers with a Tm of 60°C or use qPCR primer design software
Amplification in No-RT control	RNA is contaminated with genomic DNA	• Treat sample with DNaseI • Redesign amplicon to span exon-exon junction

Ordering Information

NEB #	PRODUCT	SIZE
E3005S/L	Luna Universal One-Step RT-qPCR Kit	200/500 reactions
E3005X	Luna Universal One-Step RT-qPCR Kit	1,000 reactions
E3005E	Lune Universal One-Step RT-qPCR Kit	2,5000 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
M0372S/L	Antarctic Thermolabile UDG	100/500 units
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
M3003S/L	Luna Universal qPCR Master Mix	200/500 reactions
M3003X	Luna Universal qPCR Master Mix	1,000 reactions
M3003E	Luna Universal qPCR Master Mix	2,500 reactions
M3004S/L	Luna Universal Probe qPCR Master Mix	200/500 reactions
M3004X	Luna Universal Probe qPCR Master Mix	1,000 reactions
M3004E	Luna Universal Probe qPCR Master Mix	2,500 reactions

Revision History

REVISION #	DESCRIPTION	DATE
1.0		11/16
1.1		12/16
1.2		7/17
1.3		6/18
2.0	Update to new manual format	1/20
3.0	Update legal text	3/20
4.0	Update legal text	10/20

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