INSTRUCTION MANUAL

Luna® Universal Probe qPCR Master Mix

NEB #M3004S/L/X/E

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

Version 3.0_3/20

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Product Format/Storage

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 master 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.

This product contains the Luna Universal Probe qPCR Master Mix, provided in volumes sufficient for preparation of up to 200 reactions (NEB #M3004S), 500 reactions (NEB #M3004L) or 1,000 reactions (NEB #M3004X) in 1 ml aliquots, or 2,500 reactions (NEB #M3004E) in a 25 ml bottle.

Required Materials Not Included

Target-specific primers

Fluorescently-labeled probe

Nuclease-free water

qPCR instrument

qPCR plates and seals

PCR strip tubes or microcentrifuge tubes

Introduction

Probe-based quantitative PCR (qPCR) uses real-time fluorescence released 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 significantly detectable 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.

The NEB Luna Universal Probe qPCR Master Mix is a 2X reaction mix optimized for real-time qPCR detection and quantitation of target DNA sequences using hydrolysis probes. It contains Hot Start *Taq* DNA Polymerase and has been 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). It also features dUTP for carryover prevention and a non-fluorescent, visible dye to monitor reaction setup. This dye does not spectrally overlap fluorophores commonly used for qPCR and will not interfere with real-time detection.

The master mix formulation is supplied at 2X concentration and contains all PCR components required for amplification and quantitation of DNA except primers/probes and DNA template. Genomic DNA or cDNA of interest can be quantitated with Luna qPCR and existing as well as commercial qPCR assay primer/probe sequences can be used.

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

- qPCR is a sensitive DNA detection method. Proper sterile technique and careful pipetting should be used to avoid DNA contamination and ensure accurate quantitation results.
- Ensure that all components are thawed and mixed prior to use. Once thawed, place on ice. For best results, reactions should be kept on ice prior to thermocycling.
- We recommend running triplicate reactions for each sample. This permits exclusion of outlier traces 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.
- Choose the detection channel of the qPCR instrument that corresponds with the fluorophore label of the target-specific probe present in the array. If using FAM on some instruments, choosing the single channel collection results in faster experiment times.
- 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. HPLC or PAGE purification may be helpful for assays that require increased sensitivity.

Luna Universal Probe qPCR Master Mix Protocols

Before Use

- *Prepare DNA or cDNA of interest using desired DNA extraction and purification method*
- *Make dilutions of DNA or cDNA 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 Probe qPCR Master Mix	10 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
Template DNA	variable	< 100 ng
Nuclease-free Water	to 20 µl	

1. Thaw Luna Universal Probe qPCR Master 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, plus 10% overage and prepare assay mix of all components except DNA 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 DNA templates 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 a plate read is included at the end of the extension step.

   Confirm the appropriate detection channel is selected for the fluorophore used in the assay.

   We recommend using the “Fast” cycling profile where applicable (e.g., Applied Biosystems StepOnePlus®, QuantStudio®, 7500 Fast instruments).

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	40–45
Extension	60°C	30 seconds (+ plate read)	

Data Analysis and Expected Results

Analyze data according to real-time instrument manufacturer instructions.

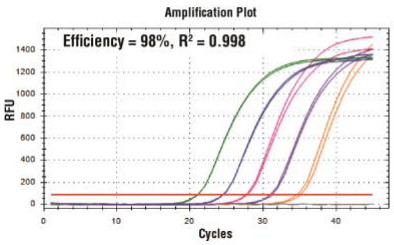
Please refer to the MIQE guidelines [Bustin, S.A., Benes, V., et al. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem., 55(4): 611−622.] for a full discussion of qPCR data analysis appropriate for peer-reviewed publication.

Briefly, this includes the following steps:

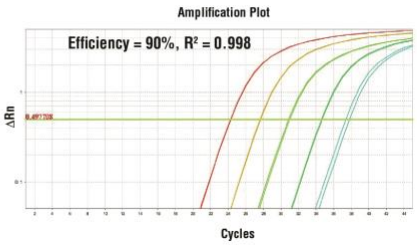
1. Determine the efficiency of the standard curve by plotting the log of the input concentration against the C_q. This can be done automatically in most qPCR instrument software packages or via the NEB online qPCR tool (accessible through NEBiocalculator® at NEBiocalculator.neb.com). The linear fit of 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 difference between the template containing and non-template control reactions. A difference in C_q of 3 or greater should be observed..
4. Evaluate any unknown samples with respect to appropriate standard curves or control samples, taking any dilution factors into account.

Brief examples of expected results:

Quantitation results in linear phase view



Quantitation results in log phase view



Usage Notes:

Assay 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 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). For cDNA targets, it is advisable to design primers across known splicing sites in order to prevent amplification from genomic DNA. Conversely, primers designed to target intronic regions can ensure amplification exclusively from genomic DNA.

Primer and Probe Concentration

For most targets, a final concentration of 400 nM for each primer will provide optimum performance. If needed, primer concentrations can be optimized between 200–900 nM. Probe should be included at 200 nM for best results. Probe concentration can be optimized in the range of 100–500 nM if optimization of performance or target fluorescence level is desired.

Multiplexing

To detect or quantitate multiple targets in the same Luna reaction, select different fluorophores corresponding to separate detection channels of the real-time instrument. Include 400 nM of forward and reverse primer and 200 nM probe for each target to be detected in the reaction, and adjust concentrations if necessary based on performance (primer 200–900 nM, probe 100–500 nM). When loading 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. For ROX-dependent instruments, avoid ROX-labeled probes. The functionality of the primer and probe sets should be tested individually before attempting a multiplex reaction. When determining

which fluorophores to include in a multiplex reaction, be sure to choose compatible reporter dyes and quenchers that have well separated fluorescence spectra or exhibit minimal overlap.

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 (including the use of longer extension times), for targets that exceed that range.

Template Preparation and Concentration

Luna qPCR is compatible with DNA samples prepared through typical nucleic acid purification methods. Prepared DNA 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 by dilution into either TE or water.

Generally, a useful concentration of standard and unknown material will be in the range of 10^6 copies to 1 copy. For gDNA samples from large genomes (e.g., human, mouse) a range of 50 ng–1 pg of gDNA is typical. For small genomes, adjust as necessary using 10^6–1 copy input as an approximate range. Note that for single copy dilutions, some samples will contain multiple copies and some will have none, as defined by the Poisson distribution.

For cDNA, use the product of a reaction containing 1 µg–0.1 pg starting RNA. cDNA does not need to be purified before addition to the Luna reaction but should be diluted at least 1:10 into the qPCR.

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. The Luna Universal Probe qPCR Master Mix is 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

qPCR is an extremely sensitive method, and contamination in new 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, the Luna Universal Probe qPCR Master Mix contains a mixture of dUTP/dTTP that results in the incorporation of dU into the DNA product during amplification. Pretreatment of 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 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 the hot start nature of the polymerase, it is not necessary to preheat the thermocycler prior to use or set up reactions on ice.

For 96-well plates, we recommend a final reaction volume of 20 µl. 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/E3004.

PROBLEM	POSSIBLE CAUSE(S)	SOLUTION(S)
qPCR traces show low or no amplification	Reagent omitted from qPCR assay Reagent added improperly to qPCR assay	• Verify all steps of the protocol were followed correctly
	Incorrect cycling protocol	• Refer to the proper qPCR cycling protocol in this user manual
	Incorrect channel selected for the qPCR thermal cycler	• Verify correct optical settings on the qPCR instrument
	Reagents are contaminated or degraded	• Confirm the expiration dates of the kit reagents • Verify proper storage conditions provided in this user manual • Rerun the qPCR assay with fresh reagents • Confirm template input amount
Inconsistent qPCR traces for triplicate data	Improper pipetting during 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 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)
DNA standard curve has a poor correlation coefficient/ efficiency of the DNA standard curve falls outside the 90-110% range	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 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
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	• 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	• Redesign primers with a Tm of 60°C or use qPCR primer design software

Ordering Information

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

Revision History

REVISION #	DESCRIPTION	DATE
1.0		11/16
1.1		12/16
1.2		7/17
1.3		9/17
2.0	Update to new manual format	1/20
3.0	Update legal text	3/20

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