INSTRUCTION MANUAL

LunaScript® Multiplex One-Step RT-PCR Kit

NEB #E1555S/L

50/250 reactions

Version 1.0_8/21

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Kit Components

This product should be stored at –20°C and has a shelf-life of 18 months when stored properly. The LunaScript Multiplex One-Step RT-PCR Reaction Mix remains frozen at -20°C and is stable for at least 30 freeze-thaw cycles. Thaw the frozen components at room temperature and keep all the components on ice or at 4°C during use. Store materials at -20°C after use.

LunaScript Multiplex One-Step RT-PCR Enzyme Mix (25X)

LunaScript Multiplex One-Step RT-PCR Reaction Mix (5X)

Nuclease-free Water

Required Materials Not Included

RNA template

Gene-specific RT-PCR primers

Thin-walled, nuclease-free PCR tubes/plates or microcentrifuge tubes

Thermal cycler

Vortex mixer

Tabletop microcentrifuge

Introduction

The LunaScript Multiplex One-Step RT-PCR Kit offers a streamlined protocol for cDNA synthesis and PCR amplification in a single reaction. The 5X reaction mix contains a reaction buffer supplemented with dNTPs and is optimized for robust multiple target detection in a simple workflow. The 25X enzyme mix features Luna® WarmStart® Reverse Transcriptase and Q5® Hot Start High-Fidelity DNA Polymerase. This dual-temperature control of enzyme activities enables room temperature reaction setup; preassembled reactions are stable at room temperature for up to 24 hours. The kit has robust multiplex target amplification capacity and enables various applications such as diagnostics, pathogen detection, and viral genome sequencing (including the ~50 amplicons per reaction used in ARTIC SARS-CoV-2 sequencing protocols).

General Tips and Considerations

Template RNA

- This kit is compatible for use with purified total RNA, mRNA, viral RNA or *in vitro* transcribed RNA.
- High-quality RNA is essential for robust target detection. RNA samples should be free of salts (e.g., Mg^{++}, guanidinium salts), organics (e.g., phenol and ethanol) and RNase contamination.
- Contaminating genomic DNA may generate false positive amplification and can be removed with DNase I treatment. When feasible, it is advisable to design primers across known RNA splicing sites to prevent amplification from genomic DNA. A no-RT control reaction can be performed to confirm the PCR product is the result of cDNA amplification.

PCR Primers

- Gene-specific primers should be used with the LunaScript Multiplex One-Step RT-PCR Kit. Random hexamers are generally not compatible with this product.
- When feasible, design primers across known RNA splicing sites to prevent amplification from genomic DNA.
- The use of PCR primer design software (e.g., Primer3) maximizes the likelihood of amplification success while minimizing nonspecific amplification and primer dimers.
- For multiplex amplification, it is recommended that the primers have a Tm within 5°C of each other. Avoid self-complementarity or inter-primer complementarity at the 3′ end.
- The final primer concentration is typically 0.5 µM per primer (1 µM total) for single-plex RT-PCR. For multiplex PCR, a total primer concentration of 1–2 µM (final) is recommended for reactions with up to 15 targets. For reactions with more targets, it may be necessary to empirically determine the appropriate primer concentration, but typically, a total concentration of 1–4 µM (final) is recommended.
- When looking for even amplicon coverage, design primers to create similar-sized amplicons. This will reduce complications due to amplification efficiencies based on size.

Amplicon Length

- For single-plex RT-PCR, the recommended amplicon length is ≤ 3 kb.
- For multiplex RT-PCR, the optimal amplicon length is between 100–1500 bp.

One-Step RT-PCR Cycling Conditions

- cDNA Synthesis
    - RT temperature: the Luna WarmStart Reverse Transcriptase can perform cDNA synthesis in the temperature range of 50°C to 65°C, with 55°C as the optimal temperature for most applications. To ensure the best performance and full WarmStart activation, avoid using an RT temperature below 50°C.
    - RT time: a 10 minute RT reaction time is sufficient for most applications. Increasing the RT time to up to 20 minutes may increase yields when the final primer concentration of each primer is less than 0.1 µM.
- cDNA Amplification by PCR:
    - Annealing temperature: use of the NEB Tm Calculator is highly recommended as optimal annealing temperatures tend to be higher for Q5 Hot Start High-Fidelity DNA Polymerase than for other polymerases. Alternative online calculators may underestimate the optimal annealing temperature. When performing multiplex RT-PCR, use the annealing temperature calculated for the amplicon with the lowest annealing temperature.
    - Annealing time: for single-plex RT-PCR, a 10–20 second annealing time is recommended; for multiplex RT-PCR, a longer annealing time is needed for optimal results. For example, a 1 minute annealing time is sufficient for a 10-plex RT-PCR assay when the final total primer concentration is 1 µM.
    - Extension: the recommended extension temperature is 72°C. Extension times are generally 20–30 seconds per kb. For amplification of more than 10 targets, an extension of 1 minute per kb at 68°C may be beneficial. If using primers with annealing temperatures ≥ 63°C, a 2-step thermal cycling protocol (combining annealing and extension into one step at the calculated annealing temperature) is possible.

- Cycle numbers: cycle number will vary depending on downstream detection methods. For agarose gel electrophoresis, 40 cycles are generally sufficient. For more sensitive detection methods, fewer PCR cycles are possible. For example, 35 PCR cycles are sufficient to generate PCR products for downstream NGS library preparation (Oxford Nanopore Technologies® or Illumina®) from as low as 10 copies of SARS-CoV-2 synthetic RNA.

LunaScript Multiplex One-Step RT-PCR Kit Protocol

1. Thaw the frozen components at room temperature. After thawing completely, mix the Reaction Mix thoroughly.*
2. Briefly centrifuge all components to collect liquid to the bottom of the tubes, then place on ice.
3. Prepare reactions as described below:

COMPONENTS	25 µl REACTION	FINAL CONCENTRATION
LunaScript Multiplex One-Step RT-PCR Reaction Mix (5X)	5 µl	1X
LunaScript Multiplex One-Step RT-PCR Enzyme Mix (25X)	1 µl	1X
Gene-specific Primer	variable	1–4 µM total**
RNA Template	variable	up to 1 µg
Nuclease-free Water	to 25 µl	–

* Precipitates may be visible upon thawing. To ensure optimal performance, resuspend completely by pipetting up and down, inverting, or by vortexing then briefly centrifuging to collect all liquid to the bottom of the tube.

** The final primer concentration is typically 0.5 µM per primer (1 µM total) for single-plex RT-PCR. For multiplex RT-PCR, a total primer concentration of 1–2 µM (final) is recommended for reactions with up to 15 targets. For reactions with more targets, it may be necessary to empirically determine the appropriate primer concentration but typically, a total concentration of 1–4 µM (final) is recommended.

Optional: No-RT Negative Control Reaction

Prepare the no-RT reaction as in Step 3 above, leaving out the RNA sample. In a thermal cycler with a heated lid, heat the reaction at 98°C for 1 minute to inactivate the Luna RT, then let the reaction cool to 25°C. When cool, transfer the reaction to ice and add the RNA sample. Proceed to Step 4.

4. Incubate the reactions in a thermal cycler with the following cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Reverse Transcription	55°C*	10 minutes	1
RT Inactivation/ Initial Denaturation	98°C	1 minute	1
Denaturation	98°C	10 seconds	35–40
Annealing	60–72°C**	10–60 seconds***	
Extension	72°C	20–30 second/kb	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

* A 55°C RT step temperature is optimal for Luna WarmStart Reverse Transcriptase. To ensure best performance and full WarmStart activation, avoid using an RT temperature below 50°C.

** Use of the NEB Tm Calculator is highly recommended as optimal annealing temperatures tend to be higher for Q5 Hot Start High-Fidelity DNA Polymerase than for other polymerases. Alternative online calculators may underestimate the optimal annealing temperature.

*** An annealing time of 10–20 seconds is recommended for single-plex reactions; it should be increased when performing multiplex RT-PCR. For example, a 1 minute annealing time is generally sufficient for a 10-plex RT-PCR assay. When the final concentration of each primer is less than 0.05 µM, longer extension time may be needed.

5. The amplified cDNA product can be stored overnight at 4°C or at -20°C for long-term storage, before proceeding to downstream applications. 5–10 µl of the PCR product can be directly examined by agarose gel electrophoresis. The product can also be cloned using the NEB® PCR Cloning Kit (NEB #E1202). For downstream NGS library construction, a cleanup step with SPRI® beads is recommended, using the manufacturer's stated protocol.

Troubleshooting Guide

Note: For additional assistance please refer to product FAQs at www.neb.com/E1555.

PROBLEM	POSSIBLE CAUSE(S)	SOLUTION(S)
Low Product Yield	Poor RNA Quality	• Check the integrity of the RNA using BioAnalyzer® or denaturing agarose gel electrophoresis (1). • RNA should have a minimum A_{260}/A_{280} ratio of 1.8 or higher. Ethanol precipitation followed by a 70% ethanol wash can remove most contaminants such as EDTA and guanidinium. Precipitation with lithium chloride can remove polysaccharides (1). • Phenol/chloroform extraction and ethanol precipitation can remove contaminant proteins such as proteases (1). • Repurify RNA samples using column-based methods e.g., Monarch® Total RNA Miniprep Kit or remove the impurity from the RNA samples using the Monarch RNA Purification Kit.
	Insufficient quantity of starting material	• Perform an RNA input titration to identify an optimal input amount.
	Primer concentration too low	• Perform a primer concentration titration targeting a final total primer concentration between 1–4 µM.
Nonspecific Product Bands	Poor RNA integrity or purity	• Ensure the purity and quality of the RNA starting material.
	Primer-dimer formation	• Check primer sets for inter-complementarities. • Use proper annealing temperature calculated by the NEB Tm calculator.
	Non-specific primer binding	• Redesign primers to ensure specific primer binding to targets.
	Amplification of genomic DNA	• Design primers spanning exons. Treat the RNA samples with DNase I to remove genomic DNA contamination.
Positive signals in the No RT Controls	DNA contamination in the RNA templates	• Perform DNase I treatment to remove DNA contaminants. • Design PCR primers spanning exons.
Expected target(s) detected in the NTC reactions	Contamination present in the reagents or process	• Follow good laboratory PCR practices. • Replace the contaminated reagent(s).
Not all PCR products are amplified/uneven amplification	Primer design not optimal	• Redesign primers for the failed amplicons if single-plex RT-PCR also fails using these primers.
	Suboptimal amplicon length	• Keep the amplicon length at 100–1500 bp range.
	Primer concentration not optimal for the failed/underperforming amplicons	• Increase the primer concentration for the failed/underperforming amplicons in the primer pool.

Not all PCR products are amplified/uneven amplification (continued)	Suboptimal RT-PCR reaction conditions	• Optimize the cycling conditions following the guidelines in the General Tips and Consideration Section.

Reference

1. Green, M.R. and Sambrook, J. (2012) *Molecular Cloning: A Laboratory Manual* (4th Ed.) Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Ordering Information

NEB #	PRODUCT	SIZE
E1555S/L	LunaScript Multiplex One-Step RT-PCR Kit	50/250 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
T2010S	Monarch Total RNA MiniPrep Kit	50 preps
T2030S/L	Monarch RNA Cleanup Kit (10 µg)	10/100 preps
T2040S/L	Monarch RNA Cleanup Kit (50 µg)	10/100 preps
T2050S/L	Monarch RNA Cleanup Kit (500 µg)	10/100 preps
M0303S/L	DNase I (RNase-free)	1,000/5,000 units
E7805S/L	NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina	24/96 reactions
E7645S/L	NEBNext Ultra II DNA Library Prep Kit for Illumina	24/96 reactions
E7546S/L	NEBNext Ultra II End Repair/dA-Tailing Module	24/96 reactions
E7595S/L	NEBNext Ultra II Ligation Module	24/96 reactions
E6440S/L	NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs)	96/384 reactions

Revision History

REVISION #	DESCRIPTION	DATE
1.0		N/A

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