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Instruction Manual

LUX™ Fluorogenic Primers

For real-time PCR and RT-PCR

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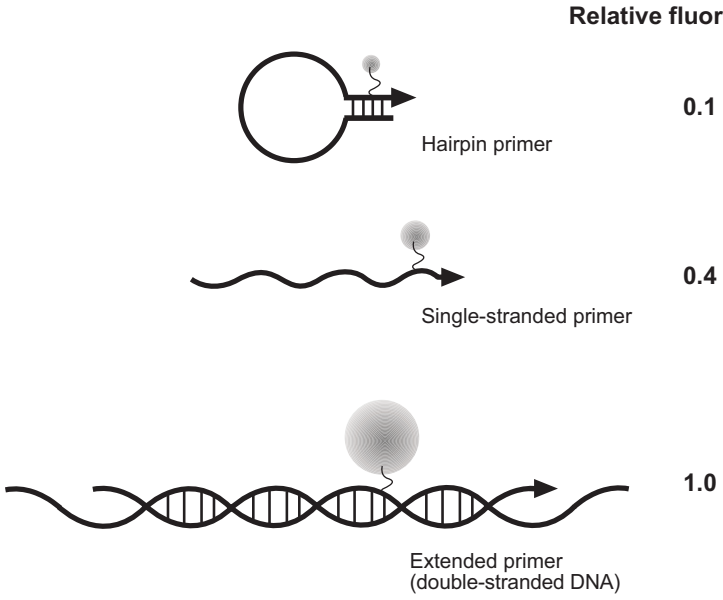
Introduction

Overview

LUX™ (Light Upon eXtension) Primers are an easy to use, highly sensitive, and efficient method for performing real-time quantitative PCR (qPCR) and RT-PCR (qRT-PCR). Each primer pair in the LUX™ system includes a fluorogenic primer with a fluorophore attached to its 3′ end and a corresponding unlabeled primer. The fluorogenic primer has a short sequence tail of 4–6 nucleotides on the 5′ end that is complementary to the 3′ end of the primer. The resulting hairpin secondary structure provides optimal quenching of the fluorophore (see the figure below). When the primer is incorporated into double-stranded DNA during PCR, the fluorophore is dequenched and the signal increases by up to 10-fold.

LUX™ Primers combine high specificity with simple design and streamlined protocols. LUX™ Primers require no special probes or quenchers and are compatible with melting curve analysis of real-time qPCR products, which allows for the differentiation of amplicons and primer dimer artifacts by their melting temperatures. LUX™ Primers are available with two different reporter dyes, which provides for multiplexing capability. You can custom-design LUX™ Primers™ from a target DNA sequence using Web- or desktop-based software, or order predesigned and validated Certified LUX™ Primer Sets for Housekeeping Genes.

LUX™ Primer Reaction



Labeling

Each fluorogenic LUX™ primer is labeled with one of two reporter dyes—FAM (6-carboxy-fluorescein) or JOE (6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein).

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Applications

LUX™ Primers can be used in real-time quantitative PCR and RT-PCR to quantify 100 or fewer copies of a target gene in as little as 1 pg of template DNA or RNA. They have a broad dynamic range of 7–8 orders. See the guidelines and sample protocols for qPCR on pages 7–10 and guidelines and sample protocols for qRT-PCR on pages 12–20.

Multiplex applications use separate FAM and JOE-labeled primer sets to detect two different genes in the same sample. Typically, a custom-designed FAM-labeled primer set would be used to detect the gene of interest, and a JOE-labeled Certified LUX™ Primer Set would be used to detect a housekeeping gene as an internal control. See the optimization guidelines for multiplex qPCR on page 11.

Instrument Compatibility

LUX™ Primers are compatible with a wide variety of real-time qPCR instruments, including but not limited to the ABI PRISM® 7700, 7000, and 7900 and GeneAmp® 5700; the Bio-Rad iCycler™; the Stratagene Mx4000™ and Mx3000™; the Cepheid Smart Cycler®; the Corbett Research Rotor-Gene; and the Roche LightCycler®.

At a minimum, the real-time qPCR instrument should:

- Detect fluorescence at each PCR cycle
- Excite and detect FAM-labeled LUX™ Primers near their excitation/emission wavelengths of 490/520 nm, and/or
- Excite and detect JOE-labeled LUX™ Primers near their excitation/emission wavelengths of 520/550 nm

Refer to the specific instrument's user manual for operating instructions.

Designing and Ordering Custom LUX™ Primers

LUX™ Designer Primer Design Software

To design and order custom LUX™ Primers for your genes of interest, visit the Invitrogen LUX™ Web site at **www.invitrogen.com/LUX** and follow the link to the **LUX™ Designer** software. The software is available as either a Web-based application or a Microsoft® Windows®-compatible download. Follow the step-by-step instructions in the software to submit your target sequence and generate primer designs.

LUX™ Designer will automatically generate one or more primer designs based on each sequence you submit and the selected design parameters. The design software includes algorithms to minimize primer self-complementarity and interactions between primers. It also assigns rankings to the generated designs—based on primer melting temperature, hairpin structure, self-annealing properties, etc.—to aid in selection.

When the designs have been generated, you can review them, select a design, select the fluorophore labels, and place your order.

Guidelines for Submitting a Target Sequence

When you submit a target sequence containing your gene of interest, keep in mind the following design criteria:

- The optimal amplicon length for real-time qPCR ranges from 80 to 200 bases. You can specify a minimum, optimal, and maximum amplicon length when you submit the sequence.
- The target sequence should be at least 10 bases longer than the minimum amplicon size you select. **The longer the sequence, the more likely that an optimal primer design can be developed**.
- The sequence must contain only standard IUPAC (International Union of Pure and Applied Chemistry) letter abbreviations.
- When you select the design parameters, the default melting temperature range is 60–68°C. Do not change this default unless the design engine finds no primers in this range. For primers in this range, PCR annealing temperatures from 55° to 64°C are appropriate.

When you first submit a sequence, the Disable Score-Based Rejection checkbox should *not* be checked; the resulting scores provide an important measure of primer suitability. **Scores in the range of 0.0–4.0 are acceptable.** If no primers with a score of 4.0 or lower can be generated from a sequence, you can disable score-based rejection and redesign the primers. See the LUX™ Designer **Help** for additional guidance.

Selecting a Primer Design

After you submit your sequence, LUX™ Designer will first generate one or more designs for the labeled primer. The labeled primer can be either the forward or the reverse primer. After you select a design for the labeled primer, you will be prompted to select a design for the corresponding unlabeled primer.

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Designing and Ordering Custom LUX™ Primers, Continued

Selecting Labels

After you have selected a primer set (labeled and unlabeled) for a particular sequence, you can specify the particular label and synthesis scale. Custom LUX™ Primers are provided in 50 nM or 200 nM synthesis scale.

When selecting labels in a multiplex reaction, we recommend using the FAM label for your gene of interest and the JOE label for the housekeeping gene that you will use as the internal control. Certified LUX™ Primer Sets for Housekeeping Genes are recommended for the JOE-labeled control gene.

Placing the Order

After you have selected the label and synthesis scale, you can submit your order to Invitrogen using the Web site or by e-mail or fax. Each primer order will be shipped directly from Invitrogen's Custom Primer Facilities. Labeled primers are supplied in an amber tube; unlabeled primers are supplied in a clear tube.

Each primer ordered from Invitrogen's Custom Primer Facilities comes with a Certificate of Analysis (COA) verifying the amount and sequence.

Product Qualification

Custom LUX™ Primers are tested post-synthesis by optical density (OD) ratio measurements and mass spectroscopy to ensure efficient dye labeling and correct molecular weight and composition.

See the Certificate of Analysis shipped with each primer for more information.

Storing and Reconstituting Primers

Primer Storage and Stability

Store primers at –20°C in the dark. LUX™ Primers are stable for:

- >12 months when stored at –20°C in lyophilized form.
- >6 months when stored at –20°C in solution.

Stability can be extended by storing at –70°C.

Important

Be careful to minimize the exposure of labeled LUX™ Primers to direct light, as this can reduce their fluorescent intensity.

Reconstituting Primers

Custom LUX™ Primers are provided lyophilized in 50-nmole or 200-nmole synthesis scale. To reconstitute primers, centrifuge the tube for a few seconds to collect the oligonucleotide in the bottom of the tube. Carefully open, add an appropriate volume of TE buffer or ultrapure water, close the tube, rehydrate for 5 minutes, and vortex for 15 seconds.

We recommend that you rehydrate primers at concentrations greater than 10 µM. To prepare a 100 µM primer stock solution, multiply the primer amount in nmoles by ten to determine the volume of diluent in µl.

After reconstitution, store the primer stock at –20°C in the dark, where it will be stable for 6 months or more.

Certified LUX™ Primer Sets for Housekeeping Genes

Certified LUX™ Primer Sets for Housekeeping Genes

Certified LUX™ Primer Sets for Housekeeping Genes are predesigned primer sets for genes that are commonly used as internal controls for normalizing real-time qRT-PCR experiments. These primer sets have been optimized and functionally validated to provide accurate, reproducible results using standard LUX™ protocols. They are supplied ready to use in TE buffer.

Each Certified LUX™ Primer Set includes a FAM- or JOE-labeled LUX™ primer and a corresponding unlabeled primer. Each primer (labeled and unlabeled is supplied at 100 µl and a concentration of 10 µM. Available sets are listed below. For additional information, visit www.invitrogen.com/LUX.

Product	GenBank Accession no.	Forward/ Reverse Label	Cat. no. FAM label	Cat. no. JOE label	Relative expression	CDS Location	PCR Product Size Range
Human genes							
18S rRNA	X03205	Forward	115HM-01	115HM-02	++++	n/a	101-150 bp
hβ-ACTIN	NM_001101	Forward	101H-01	101H-02	+++	Exons 2/3	101-150 bp
hATPSase	NM_001686	Forward	108H-01	108H-02	+++	n/a	101-150 bp
hB2M	NM_004048	Forward	113H-01	113H-02	+++	Exons 1/2	101-150 bp
hGAPDH	NM_002046	Forward	100H-01	100H-02	+++	Exons 4/5	151–200 bp
hPGK1	NM_000291	Forward	109H-01	109H-02	+++	n/a	50-100 bp
hPPIA	NM_021130	Forward	106H-01	106H-02	+++	Exons 2/3	50-100 bp
hRPL4	NM_000968	Reverse	103H-01	103H-02	+++	Exons 8/9	101-150 bp
hEEF1G	NM_001404	Forward	107H-01	107H-02	++	n/a	50-100 bp
hHPRT1	NM_000194	Reverse	105H-01	105H-02	++	Exons 5/6	50-100 bp
hSDHA	NM_004168	Forward	102H-01	102H-02	++	Exons 12/13	50-100 bp
hTFRC	NM_003234	Forward	111H-01	111H-02	++	Exons 10/11	101-150 bp
hGUS	NM_000181	Forward	112H-01	112H-02	+	Exons 7/8	101-150 bp
hHMBS	NM_000190	Forward	110H-01	110H-02	+	Exons 2/3	50-100 bp
hTBP	NM_003194	Forward	104H-01	104H-02	+	Exons 3/4	101-150 bp
hUBC	NM_021009	Forward	114H-01	114H-02	+	n/a	50-100 bp
Mouse/rat genes							
18S rRNA	X03205	Forward	115HM-01	115HM-02	++++	n/a	101-150 bp
mβ-ACTIN	NM_007393	Forward	101M-01	101M-02	+++	Exons 2/3	101-150 bp
mB2M	X01838	Forward	113M-01	113M-02	+++	n/a	50-100 bp
mEEF1G	AF321126	Forward	107M-01	107M-02	+++	n/a	101-150 bp
mGAPDH	NM_008084	Forward	100M-01	100M-02	+++	Exons 4/5	151–200 bp
mPGK1	NM_008828	Forward	109M-01	109M-02	+++	Exons 1/2	101-150 bp
mPPIA	NM_008907	Reverse	106M-01	106M-02	+++	Exons 1/2	50-100 bp
mRPL4	NM_022510	Forward	103M-01	103M-02	+++	Exons 2/3	151-200 bp
mHPRT1	NM_013556	Forward	105M-01	105M-02	++	Exons 6/7	50-100 bp
mSDHA	AF095938	Forward	102M-01	102M-02	++	Exons 6/7	50-100 bp
mATPSase	NM_016774	Forward	108M-01	108M-02	+	n/a	50-100 bp
mGUS	NM_010368	Forward	112M-01	112M-02	+	Exons 7/8	50-100 bp
mHMBS	XM_129404	Reverse	110M-01	110M-02	+	Exons 4/5	50-100 bp
mTBP	NM_013684	Forward	104M-01	104M-02	+	Exons 3/4	101-150 bp
mTFRC	NM_011638	Forward	111M-01	111M-02	+	Exons 2/3	101-150 bp
***Drosophila* genes**							
d18S rRNA	AY037174	Reverse	115D-01	115D-02	++++	n/a	101-150 bp
dActin	NM_079486	Forward	101D-01	101D-02	+++	Exons 2/3	50–100 bp

Real-Time qPCR

Introduction

Real-time qPCR uses genomic DNA, cDNA, or plasmid DNA as starting material. This section provides guidelines and example protocols for performing real-time qPCR using LUX™ Primers.

Template Specifications

The target template for real-time qPCR is linear single-stranded or double-stranded DNA, cDNA, or circular DNA (such as plasmids) that has been linearized. The amount of DNA typically ranges from 10^2 to 10^7 copies or 1 pg to 10 µg of template.

See page 12 for instructions on generating cDNA using reverse transcription as part of two-step real-time qRT-PCR.

Primer Concentration

For optimal PCR conditions, primer titrations of 50–500 nM per primer are recommended. The sample reactions on pages 9–10 use 200 nM of each primer, equivalent to 1 µl of a 10-µM primer solution in a 50-µl reaction.

Magnesium Concentration

The optimal Mg^{++} concentration for a given target/primer/polymerase combination can vary between 1 mM and 10 mM, but is usually in the range of 3 mM. See the sample reactions on pages 9–10.

dNTP Concentration

The optimal concentration of dATP, dCTP, dGTP, and dTTP is 200 µM each. If dUTP is used in place of dTTP, its optimal concentration is 400 µM.

DNA Polymerase

We recommend using a "hot-start" DNA polymerase, preferably one that has been optimized for real-time qPCR. Platinum® Quantitative PCR SuperMix UDG (Catalog no. 11730-017) is a 2X-concentrated, ready-to-use mixture containing all components except primers and template. It uses Platinum® *Taq* DNA polymerase and has been specifically formulated to provide optimal performance in real-time qPCR systems.

ROX Reference Dye

We recommend using ROX Reference Dye (Cat. no. 12223-023) to normalize the fluorescent reporter signal in real-time qPCR for instruments that are compatible with this option. ROX Reference Dye can be used to adjust for non-PCR-related fluctuations in fluorescence between reactions, and provides a stable baseline in multiplex reactions. It is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester (25 µM) in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20.

ROX is supplied at 50X concentration. Add 1 µl of ROX for every 50 µl of reaction volume. For convenience and to reduce pipetting errors, you can premix a solution of ROX and Platinum® Quantitative PCR SuperMix-UDG. Add 1 µl of ROX for every 25 µl of SuperMix-UDG. Store mixture at either -20°C or 4°C in the dark.

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Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) is required in qPCR reactions on the Roche LightCycler® because the glass capillaries in the LightCycler® have a high surface-to-volume ratio and the glass surface binds molecules like *Taq* DNA polymerase, effectively removing them from the reaction. The addition of BSA blocks this surface binding.

Nonacetylated BSA is strongly recommended because acetylated BSA will inhibit PCR at the concentrations required in LightCycler® reactions. This inhibition is most likely due to the transfer of acetyl groups to essential components of the PCR, like the *Taq* DNA polymerase.

To ensure that the BSA does not contain RNA or DNA, we recommend using ultrapure, molecular biology-grade nonacetylated BSA from Panvera/Invitrogen (Cat. nos. P2489 and P2046).

Melting Curve Analysis

Melting curve analysis is strongly recommended during qPCR to identify the presence of primer dimers and analyze the specificity of the reaction. Program your real-time instrument to perform this analysis after thermocycling.

Melting curve analysis identifies the change in fluorescent signal that occurs as double-stranded DNA (dsDNA) dissociates or "melts" into single-stranded DNA. By identifying the temperature at which the dsDNA dissociates, you can distinguish smaller artifacts like primer dimers with a lower annealing temperature from larger amplicons with a higher annealing temperature.

The presence of primer dimers in samples containing template decreases PCR efficiency and obscures analysis and determination of cycle thresholds. The formation of primer dimers most often occurs in no-template controls, where the polymerase enzyme is essentially idle, and in this case the quantitative analysis of the template samples is not affected. Melting curve analysis of no-template controls can discriminate between primer dimers and spurious amplification due to contaminating nucleic acids in reagent components.

Instrument Settings

Follow the manufacturer's instructions for configuring your real-time qPCR instrument for use with LUX™ Primers. Note the following general settings:

- Program your instrument to perform melting curve analysis at the end of thermocycling, if this option is available.
- The quencher setting on the instrument should reflect the fact that LUX™ Primers do not contain a quencher.
- We recommend using ROX Reference Dye, if your instrument is compatible with this option. Adjust the instrument settings accordingly.

Additional guidelines and settings for specific instruments are available at www.invitrogen.com/lux; click on **Instrument Protocols**.

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Protocol for Instruments Using PCR Tubes or Plates

The following protocol uses Platinum® Quantitative PCR SuperMix-UDG with ROX reference reagent. It has been optimized for use with real-time qPCR instruments that use PCR tubes or plates. A protocol for the Roche LightCycler® is provided on the following page.

Note: The following protocol uses a 50-µl reaction volume; smaller volumes may be used, depending on the requirements of your instrument. Before proceeding, see the real-time qPCR guidelines on the previous pages. For multiplex reactions, see the guidelines on page 11.

1. To reduce well-to-well variation, prepare a Master Mix of all the reaction ingredients except template. The following table provides Master Mix volumes for one reaction and 50 reactions (scale up or down as needed):

Component	Vol/1 rxn	Vol/50 rxns
Platinum® Quantitative PCR SuperMix-UDG[1]	25 µl	1250 µl
ROX Reference Dye (optional)	1 µl	50 µl
Labeled LUX™ Primer (10 µM)	1 µl	50 µl
Unlabeled primer (10 µM)	1 µl	50 µl
Sterile distilled water[2]	to 40 µl	to 2000 µl

[1]Final concentration: 0.03 U/µl Platinum® *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 1 U UDG

[2]or use DNase/RNase Free Distilled Water (Cat. No. 10977-015).

2. Program the real-time qPCR instrument as follows:

3-Step Cycling (recommended)	2-Step Cycling (optional)
50°C, 2 min hold (UDG treatment)	50°C, 2 min hold (UDG treatment)
95°C, 2 min hold	95°C, 2 min hold
45 cycles of:	45 cycles of:
95°C, 15 s	95°C, 15 s
55°C, 30 s	60-65°C, 30-45 s
72°C, 30 s	

Melting Curve Analysis (recommended)
Refer to instrument documentation

3. Add 40 µl of the Master Mix to an optical PCR tube or each well of a 96-well PCR plate.
4. Add 10 µl of template in TE or sterile dH_2O to each reaction vessel. Cap or seal the tube/plate.
5. Gently mix and make sure that all components are at the bottom of the tube/plate wells. Centrifuge briefly if needed.
6. Place reaction in the real-time qPCR instrument and run the program. Collect and analyze results.

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Protocol for the Roche LightCycler®

The following protocol uses Platinum® Quantitative PCR SuperMix-UDG and has been optimized for the Roche LightCycler®. Consult the LightCycler® documentation for detailed instructions on preparing the capillary tubes and operating the instrument. FAM-labeled LUX™ Primers are also compatible with Roche enzyme mixes.

Note: JOE-labeled LUX™ Primers are not compatible with the current version of the LightCycler®; use FAM-labeled primers only. The following protocol uses a 20-µl reaction volume. Before proceeding, see the real-time qPCR guidelines on the previous pages.

1. To reduce well-to-well variation, prepare a Master Mix of all the reaction ingredients except template. The following table provides volumes for one reaction and 34 reactions (scale as needed):

Component	Vol/1 rxn	Vol/34 rxns
Platinum® Quantitative PCR SuperMix-UDG[1]	10 µl	340 µl
FAM-labeled LUX™ Primer (10 µM)	1 µl	34 µl
Unlabeled primer (10 µM)	1 µl	34 µl
Bovine serum albumin (5 mg/ml)[2]	1 µl	34 µl
Platinum® *Taq* DNA Polymerase[3]	0.12 µl	4 µl
Sterile distilled water[4]	to 18 µl	to 612 µl

[1]Final concentration: 0.03 U/µl Platinum® *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 1 U UDG

[2]Validated with non-acetylated Ultrapure BSA (10% solution) from Panvera (Cat. nos. P2489 and P2046).

[3]Total units of Platinum® *Taq* DNA Polymerase in the reaction is 1.2 (including 0.6 U from Platinum® Quantitative PCR SuperMix-UDG)

[4]or use DNase/RNase Free Distilled Water (Cat. No. 10977-015).

2. Set the fluorescence on the Roche LightCycler® to the F1 channel.

3. Program the instrument as follows:

Thermal Cycling

Program choice: Amplification
Analysis mode: Quantification
Cycling:
50°C, 2 min hold (UDG treatment)
95°C, 2 min hold
45 cycles of:
  94°C, 5 s
  55°C, 10 s (single acquire)
  72°C, 10 s

Melting Curve Analysis (optional)

Program choice: Melting curve
Analysis mode: Melting curves
Cycling:
95°C, 0 s
55°C, 15 sec
95°C, 0 (increase 0.1°C/s with continuous acquisition)
40°C, 0 s

4. Add 18 µl of Master Mix to each capillary tube of the LightCycler®.

5. Add 2 µl of template to each tube, and cap the tube.

6. Centrifuge the tubes at 700 × *g* for 5 seconds.

7. Place the reaction tubes in the rotor of the LightCycler® and run the program. Collect and analyze results.

Multiplex Real-Time qPCR

Multiplex Real-Time qPCR

In multiplex real-time qPCR, different sets of primers with different fluorogenic labels are used to amplify separate genes in the template DNA. Multiplexing with LUX™ Primers offers simplified PCR kinetics and increased reaction efficiency when compared with probe-based technologies, because only two oligos are used per target.

LUX™ Primers have been tested in multiplex reactions using a FAM-labeled primer set for the gene of interest and a JOE-labeled set for a housekeeping gene used as an internal control to normalize between different reactions. We recommend using Certified LUX™ Primer Sets for Housekeeping Genes for the internal control (see page 6).

Note: We recommend selecting a housekeeping gene that matches the relative expression level of your gene of interest. The relative expression levels of predesigned, certified LUX™ Primer Sets are shown on page 6.

In a standard multiplex reaction, you can include the additional primers at the same volumes and concentration as the primers in a singleplex reaction, as shown in the example mixture below:

Component	Volume
Platinum® Quantitative PCR SuperMix-UDG (2X)	25 µl
ROX Reference Dye (50X)	1 µl
Template	10 µl
Forward primer 1 (FAM label) (10 µM)	1 µl
Reverse primer 1 (10 µM)	1 µl
Forward primer 2 (JOE label) (10 µM)	1 µl
Reverse primer 2 (10 µM)	1 µl
Sterile distilled water	to 50 µl

All other reaction volumes remain the same.

Follow the thermal cycling guidelines provided in **Protocol for Instruments Using PCR Tubes or Plates** on page 9. If you have difficulty performing the multiplex reaction using these guidelines, see the optimization hints below.

Optimizing Multiplex Conditions

If you notice a decline in PCR efficiency in your multiplex real-time qPCR, you can optimize the reaction by performing the steps listed below.

Note: We recommend that you perform one optimization step and then repeat the reaction to test for efficiency before moving on to the next step:

1. Reduce the primer concentration of the gene with the highest expression levels (typically the housekeeping gene) to 1/2 the primer concentration of the other gene. For example, in a standard 50-µl reaction, you would add the primers for the less abundant gene at 1 µl each, and add the primers for the more abundant gene at 0.5 µl each.
2. Increase the $MgCl_2$ in the reaction from 3 mM to 6 mM.
3. Double the amount of polymerase enzyme (to 0.06 U/µl of reaction volume). If you are using Platinum® Quantitative PCR SuperMix-UDG, add Platinum® *Taq* DNA polymerase stand-alone enzyme (Catalog no. 10966-018) to double the amount of enzyme.
4. Increase the dNTP concentrations in the reaction to 400 µM each.

Two-Step Real-Time qRT-PCR

Introduction

Real-time qRT-PCR uses RNA as starting material in a reverse transcription reaction to generate first-strand cDNA. The cDNA is then quantified in a separate real-time qPCR reaction.

In two-step qRT-PCR, first-strand synthesis is performed, and then the reaction is transferred to a separate tube for the qPCR reaction. This section provides guidelines for two-step qRT-PCR and an optimized protocol using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit.

Note

For the real-time qPCR portion of the two-step protocol, see additional guidelines on primers, magnesium, dNTPs, ROX Reference Dye, BSA, melting curve analysis, and instrument settings on pages 7–8.

Template Specifications

The target template for two-step qRT-PCR is total RNA or mRNA. High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis and accurate quantification. Starting material can range from 10 pg to 1 µg total RNA. Then use 1 pg to 10 µg of the cDNA from the first-strand reaction in the qPCR step.

The purity and integrity of the starting RNA have a direct impact on results. RNase and genomic DNA contamination are the most common problems, and purification methods should include RNase inhibitors and DNase digestion to minimize these.

We recommend using the Micro-to-Midi Total RNA Purification System (Catalog no. 12183-018) or TRIzol® reagent (Catalog no. 15596-026) to isolate total RNA. High-quality total RNA can be purified from as little as 100 cells up to 10^7 cells or 200 mg of tissue.

Isolation of mRNA is typically not required, but can be performed using the FastTrack® 2.0 mRNA Isolation Kit (Catalog no. K1593-02).

Enzyme Specifications

For two-step qRT-PCR, we recommend using a high-specificity, high-yield reverse transcriptase such as SuperScript™ III Reverse Transcriptase and a "hot-start" DNA polymerase such as Platinum® *Taq* DNA Polymerase. The SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 and 11734-068) includes SuperScript™ III RT, Platinum® *Taq* DNA Polymerase, and all the other necessary components for two-step qRT-PCR except the RNA. See the example protocol on page 13.

For first-strand cDNA synthesis alone, we recommend the SuperScript™ III First-Strand Synthesis System for RT-PCR (Catalog no. 18080-051).

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Removing Genomic DNA from RNA Samples

We strongly recommend that you decrease the genomic DNA content in the RNA sample by performing a digest with DNase I, Amplification Grade (Catalog no. 18068-015), as described below. The DNase I digest is designed for up to 1 µg of RNA; for larger amounts of RNA, increase volumes accordingly.

Combine the following in a tube on ice:

Component	Conc.	Volume
RNA template	—	x µl
DNase reaction buffer	10X	1 µl
DNase I, Amplification Grade	1 U/µl	1 µl
DEPC-treated ddH_20		to 10 µl

1. Incubate at room temperature for 15 min.
2. Add 1 µl of 25-mM EDTA solution to the reaction mixture and incubate at 65°C for 10 min to inactivate the DNase I.

Reverse Transcription Protocol

The following protocol for generating first-strand cDNA uses components from the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 and 11734-068). The RT Enzyme Mix contains SuperScript™ III RT and RNaseOUT™. The 2X RT Reaction Mix contains oligo$(dT)_{20}$ (2.5 µM), random hexamers (2.5 ng/µl), 10 mM $MgCl_2$, and dNTPs.

Note: The *E. coli* RNase H digestion step is included to remove the RNA template from the cDNA:RNA hybrid molecule after first-strand synthesis. This has been shown to increase PCR sensitivity.

1. Combine the following kit components in a tube on ice. For multiple reactions, a master mix without RNA may be prepared:

2X RT Reaction Mix	10 µl
RT Enzyme Mix	2 µl
RNA (10 pg to 1 µg)	x µl
DEPC-treated water	to 20 µl

2. Gently mix tube contents and incubate at 25°C for 10 min.
3. Incubate tube at 42°C for 50 min.
4. Terminate the reaction at 85°C at 5 min, and then chill on ice.
5. Add 1 µl (2 U) of *E. coli* RNase H and incubate at 37°C for 20 min.

Store the reaction at –20°C until use. Proceed to the real-time qPCR protocol on the following page.

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Real-Time qPCR Protocol for Instruments Using PCR Tubes or Plates

The following real-time qPCR protocol uses components from the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 and 11734-068). It has been optimized for use with real-time qPCR instruments that use tubes or plates. See the guidelines on pages 7–8. A protocol for the Roche LightCycler® is provided on the following page.

1. To reduce well-to-well variation, prepare a Master Mix of all the reaction ingredients except template:

Component	Vol/1 rxn	Vol/50 rxns
Platinum® Quantitative PCR SuperMix-UDG[1]	25 µl	1250 µl
ROX Reference Dye (optional)	1 µl	50 µl
Labeled LUX™ Primer (10 µM)	1 µl	50 µl
Unlabeled primer (10 µM)	1 µl	50 µl
Sterile distilled water	to 45 µl	to 2250 µl

[1]Final concentration: 0.06 U/µl Platinum® *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 0.04 U/µl UDG

2. Program the real-time qPCR instrument as follows:

   Thermal Cycling
   50°C, 2 min hold (UDG treatment)
   95°C, 2 min hold
   45 cycles of:
   95°C, 15 s
   55°C, 30 s
   72°C, 30 s
   Melting Curve Analysis
   Refer to instrument documentation

3. Add 45 µl of the Master Mix to an optical PCR tube or each well of a 96-well PCR plate.

4. Add ~5 µl (10^2 to 10^7 copies or 1 pg to 10 µg) of the cDNA from the first-strand synthesis reaction (step 5, page 13) to each reaction vessel. Cap or seal the tube/plate.

5. Gently mix and make sure that all components are at the bottom of the tube/plate wells. Centrifuge briefly if needed.

6. Place reaction in the real-time qPCR instrument and run the program. Collect and analyze results.

Continued on next page

Protocol for the Roche LightCycler®

The following protocol uses components from the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 and 11734-068) and has been optimized for the Roche LightCycler®. Consult the LightCycler® documentation for detailed instructions on preparing the capillary tubes and operating the instrument. FAM-labeled LUX™ Primers are also compatible with Roche enzyme mixes.

Note: JOE-labeled LUX™ Primers are not compatible with the current version of the LightCycler®; use FAM-labeled primers only. The following protocol uses a 20-µl reaction volume. Before proceeding, see the real-time qPCR guidelines on pages 7–8.

1. To reduce well-to-well variation, prepare a Master Mix of all the reaction ingredients except template. The following table provides volumes for one reaction and 34 reactions (scale as needed):

Component	Vol/1 rxn	Vol/34 rxns
Platinum® Quantitative PCR SuperMix-UDG[1]	10 µl	340 µl
FAM-labeled LUX™ Primer (10 µM)	1 µl	34 µl
Unlabeled primer (10 µM)	1 µl	34 µl
BSA, UltraPure (5 mg/ml)	1 µl	34 µl
Platinum® *Taq* DNA Polymerase[2]	0.12 µl	4 µl
Sterile distilled water	to 18 µl	to 612 µl

[1] Final concentration: 0.06 U/µl Platinum® *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 0.04 U/µl UDG

[2]Total units of Platinum® *Taq* DNA Polymerase in the reaction is 1.2 (including 0.6 U from Platinum® Quantitative PCR SuperMix-UDG).

2. Set the fluorescence on the Roche LightCycler® to the F1 channel.

3. Program the instrument as follows:

Thermal Cycling

Program choice: Amplification
Analysis mode: Quantification
Cycling:
50°C, 2 min hold (UDG treatment)
95°C, 2 min hold
45 cycles of:
- 94°C, 5 s
- 55°C, 10 s (single acquire)
- 72°C, 10 s

Melting Curve Analysis (optional)

Program choice: Melting curve
Analysis mode: Melting curves
Cycling:
95°C, 0 s
55°C, 15 sec
95°C, 0 (increase 0.1°C/s with continuous acquisition)
40°C, 0 s

4. Add 18 µl of Master Mix to each capillary tube of the LightCycler®.

5. Add 2 µl (10^2 to 10^7 copies or 1 pg to 10 µg) of the cDNA from the first-strand synthesis reaction (step 5, page 13) to each tube, and cap the tube.

6. Centrifuge the tubes at 700 × *g* for 5 seconds.

7. Place the reaction tubes in the rotor of the LightCycler® and run the program. Collect and analyze results.

One-Step Real-Time qRT-PCR

Introduction

In one-step real-time qRT-PCR, cDNA synthesis and PCR are performed in a single reaction tube using total RNA or mRNA as starting material. The optimized enzyme mixture includes both a reverse transcriptase and a DNA polymerase. This section provides guidelines and an example protocol for performing one-step real-time qRT-PCR using LUX™ Primers. The example protocol uses the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System for superior specificity and sensitivity with LUX™ Primers.

Primer Concentration

For optimal one-step qRT-PCR, primer titrations of 50–500 nM per primer are recommended. The 50-µl sample reaction on page 19 uses 200 nM of each primer, equivalent to 1 µl of a 10 µM primer solution. Also see the note below.

In one-step qRT-PCR, the reverse primer drives the reverse transcription reaction. We have found that doubling the concentration of the reverse primer from 200 nM to 400 nM can in some cases decrease the cycle threshold for detecting a given target concentration, and thus increase sensitivity. See pages 3–4 for guidance on primer design.

Template Specifications

The target template for one-step real-time qRT-PCR is RNA—usually total cellular RNA or mRNA. The amount of template typically ranges from 1 pg to 100 ng per assay. The purity and integrity of the RNA have a direct impact on results. RNase and genomic DNA contamination are the most common problems, and purification methods should be designed to avoid these.

We recommend using the Micro-to-Midi Total RNA Purification System (Catalog no 12183-018) or TRIzol® reagent (Catalog no. 15596-026) to isolate total RNA. High-quality total RNA can be purified from as little as 100 cells up to 10^7 cells or 200 mg of tissue.

To isolate mRNA, we recommend using the FastTrack® 2.0 mRNA Isolation Kit (Catalog no. K1593-02).

Enzyme Specifications

The one-step qRT-PCR enzyme mix should contain an optimized mixture of a high-specificity, high-yield reverse transcriptase and a hot-start DNA polymerase. We recommend using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Catalog nos. 11732-020 and -088), which uses a SuperScript™ III RT/Platinum® *Taq* enzyme mix. See the sample reactions on pages 19–20.

Magnesium Concentration

The optimal $MgCl_2$ concentration for a given target/primer/polymerase combination can vary between 1 mM and 10 mM, but is usually in the range of 3 mM (see the sample reaction on page 19).

dNTP Concentration

The optimal concentration of dATP, dCTP, dGTP, and dTTP is 200 µM each. If dUTP is used in place of dTTP, its optimal concentration is 400 µM.

Continued on next page

ROX Reference Dye

We recommend using ROX Reference Dye (Cat. no. 12223-023) to normalize the fluorescent reporter signal for instruments that are compatible with this option. ROX Reference Dye can be used to adjust for non-PCR-related fluctuations in fluorescence between reactions, and provides a stable baseline in multiplex reactions. It is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester (25 µM) in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20.

ROX is supplied at 50X concentration. Add 1 µl of ROX for every 50 µl of reaction volume.

Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) is required in qPCR reactions on the Roche LightCycler® because the glass capillaries in the LightCycler® have a high surface-to-volume ratio and the glass surface binds molecules like *Taq* DNA polymerase, effectively removing them from the reaction. The addition of BSA blocks this surface binding.

Nonacetylated BSA is strongly recommended because acetylated BSA will inhibit PCR at the concentrations required in LightCycler® reactions. This inhibition is most likely due to the transfer of acetyl groups to essential components of the PCR, like the *Taq* DNA polymerase.

To ensure that the BSA does not contain RNA or DNA, we recommend using ultrapure, molecular biology-grade nonacetylated BSA from Panvera/Invitrogen (Cat. nos. P2489 and P2046).

Melting Curve Analysis

Melting curve analysis is strongly recommended during one-step qRT-PCR to identify the presence of primer dimers and analyze the specificity of the reaction. Program your real-time instrument to perform this analysis after thermocycling.

Melting curve analysis identifies the change in fluorescent signal that occurs as double-stranded DNA (dsDNA) dissociates or "melts" into single-stranded DNA. By identifying the temperature at which the dsDNA dissociates, you can distinguish smaller artifacts like primer dimers with a lower annealing temperature from larger amplicons with a higher annealing temperature.

The presence of primer dimers in samples containing template decreases reaction efficiency and obscures analysis and determination of cycle thresholds. The formation of primer dimers most often occurs in no-template controls, where the polymerase enzyme is essentially idle, and in this case the quantitative analysis of the template samples is not affected. Melting curve analysis of no-template controls can discriminate between primer dimers and spurious amplification due to contaminating nucleic acids in reagent components.

Continued on next page

Instrument Settings

Follow the manufacturer's instructions for configuring your real-time qPCR instrument for use with LUX™ Primers. Note the following general settings:

- Program your instrument to perform melting curve analysis at the end of thermocycling, if this option is available.
- The quencher setting on the instrument should reflect the fact that LUX™ Primers do not contain a quencher.
- We recommend using ROX Reference Dye, if your instrument is compatible with this option. Adjust the instrument settings accordingly.

Additional guidelines and settings for specific instruments are available at www.invitrogen.com/lux; click on **Instrument Protocols**.

Removing Genomic DNA from RNA Samples

We recommend that you decrease the genomic DNA content in the RNA sample by performing a digest with DNase I, Amplification Grade (Catalog no. 18068-015), as described below. The DNase I digest is designed for up to 1 µg of RNA; for larger amounts of RNA, increase volumes accordingly.

Combine the following in a tube on ice:

Component	Conc.	Volume
RNA template	—	x µl
DNase reaction buffer	10X	1 µl
DNase I, Amplification Grade	1 U/µl	1 µl
DEPC-treated ddH_20		to 10 µl

1. Incubate at room temperature for 15 min.
2. Add 1 µl of 25-mM EDTA solution to the reaction mixture and incubate at 65°C for 10 min to inactivate the DNase I.

To verify the absence of genomic DNA in the RNA sample, prepare a control reaction identical to the reactions on pages 19–20, using 2 U of Platinum® *Taq* DNA polymerase (Catalog no. 10966-018) in place of the SuperScript™ III RT/Platinum® *Taq* Mix.

Continued on next page

Protocol for Instruments Using PCR Tubes or Plates

The following protocol using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System has been optimized for LUX™ Primers. Further optimization may be required.

Note: Keep all components, reaction mixes and samples on ice. After assembly, transfer the reaction to a thermal cycler preheated to the cDNA synthesis temperature and immediately begin RT-PCR. We recommend performing the cDNA synthesis reaction at 50°C, but higher temperatures (up to 60°C) may be required for high GC content templates.

RNase inhibitor proteins, such as RNaseOUT™ (Catalog no. 10777-019), may be added to the reaction to safeguard against degradation of RNA.

1. The following table provides Master Mix volumes for a standard 50-µl reaction size. Note that preparation of a master mix is **crucial** in quantitative applications to reduce pipetting errors.

Component	Vol/1 rxn	Vol/100 rxns
SuperScript™ III RT/Platinum® *Taq* Mix	1 µl	100 µl
2X Reaction Mix[1]	25 µl	2500 µl
ROX Reference Dye (optional)	1 µl	100 µl
Labeled LUX™ Primer (10 µM)	1 µl	100 µl
Unlabeled primer (10 µM)[2]	1 µl	100 µl
RNaseOUT™ (optional)	1 µl	100 µl
Sterile distilled water	to 40 µl	to 4000 µl

[1]Supplied at 2X concentration: includes 0.4 mM of each dNTP and 6 mM $MgSO_4$

[2]See the **Important** note on primer concentration on page 16.

2. Program the instrument with the following thermal cycling protocol (for cDNA synthesis, use a 15-min incubation at 50°C as a starting point):

   cDNA synthesis:
   50°C for 15 min hold

   PCR:
   95°C for 2 min hold
   40–50 cycles of:
   95°C, 15 s
   60°C, 30 s

   Melting Curve Analysis (optional)
   Program according to instrument instructions

3. For each reaction, add 40 µl of the master mix to a 0.2-ml microcentrifuge tube or each well of a 96-well PCR plate on ice.

4. Add 10 µl of sample RNA (1 pg to 1 µg total RNA) to each tube/plate well, and cap or seal.

5. Gently mix and make sure that all components are at the bottom of the tube/plate wells. Centrifuge briefly if needed.

6. Place reactions in a preheated thermal cycler programmed as described above. Collect data and analyze results.

Continued on next page

Protocol for the Roche LightCycler®

The following protocol using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System has been optimized for LUX™ Primers and the Roche LightCycler®. Further optimization may be required. FAM-labeled LUX™ Primers are also compatible with Roche enzyme mixes.

Note: JOE-labeled primers are not compatible with the current version of the LightCycler®; use FAM-labeled primers only.

After assembly, transfer the reaction to a thermal cycler preheated to the cDNA synthesis temperature and immediately begin RT-PCR. We recommend performing the cDNA synthesis reaction at 50°C, but higher temperatures (up to 60ºC) may be required for high GC content templates.

RNase inhibitor proteins, such as RNaseOUT™ (Catalog no. 10777-019), may be added to the reaction to safeguard against degradation of RNA.

1. The following table provides Master Mix volumes for a standard 20-µl reaction size. Note that preparation of a master mix is **crucial** in quantitative applications to reduce pipetting errors.

Component	Vol/1 rxn	Vol/34 rxns
SuperScript™ III RT/Platinum® *Taq* Mix	0.8 µl	27.2 µl
2X Reaction Mix[1]	10 µl	340 µl
FAM-labeled LUX™ Primer (10 µM)[2]	1 µl	34 µl
Unlabeled primer (10 µM)[3]	1 µl	34 µl
Bovine serum albumin (5 mg/ml)[4]	1 µl	34 µl
Sterile distilled water	to 18 µl	to 612 µl

[1]Includes 0.4 mM of each dNTP and 6 mM $MgSO_4$
[2]In the LightCycler® reaction, the LUX™ Fluorogenic Primer must be FAM labeled.
[3]See the **Important** note on primer concentration on page 16.
[4]Validated with non-acetylated Ultrapure BSA (10% solution) from Panvera (Cat. nos. P2489 and P2046)

2. Set the fluorescence on the Roche LightCycler® to the F1 channel.
3. Program the instrument as follows:

Thermal Cycling

Program choice: Amplification
Analysis mode: Quantification
Cycling:
45ºC, 30 min hold (cDNA synthesis)
95ºC, 2 min hold
50 cycles of:
  95ºC, 5 s
  55ºC, 10 s (single acquire)
  72ºC, 10 s

Melting Curve Analysis (optional)

Program choice: Melting curve
Analysis mode: Melting curves
Cycling:
95ºC, 0 s
55ºC, 15 sec
95ºC, 0 (increase 0.1ºC/s with continuous acquisition)
40ºC, 0 s

4. Add 18 µl of Master Mix to each capillary tube of the LightCycler® on ice.
5. Add 2 µl of sample RNA (1 pg to 1 µg total RNA) to each capillary tube and cap the tube.
6. Centrifuge the tubes at 700 × *g* for 5 seconds.
7. Place the reaction tubes in the rotor of the LightCycler® and run the program. Collect and analyze results.

Troubleshooting

Problem	Cause	Solution
Signals are present in no-template controls, and/or multiple peaks are present in the melting curve graph	Template or reagents are contaminated by nucleic acids (DNA, cDNA)	Use melting curve analysis and/or run the PCR products on a 4% agarose gel in an area separate from the reaction assembly area to identify contaminants. To reduce the risk of contamination, take standard precautions when preparing your PCR reactions. Ideally, amplification reactions should be assembled in a DNA-free environment. We recommend using aerosol-resistant barrier tips.
	Amplification of PCR carryover products	Analyze the PCR product on a 4% agarose gel in an area separate from the reaction assembly area to identify contaminants. We recommend using a UDG-based carryover prevention system such as Platinum® Quantitative PCR SuperMix-UDG or the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (protocols provided on pages 9–10 and pages 12–15, respectively). Since dUTP is substituted for dTTP in the reaction cocktail, any amplified DNA will contain uracil. UDG prevents reamplification of PCR carryover products by removing uracil residues from single or double stranded DNA. dU-containing DNA that has been digested with UDG is unable to serve as template in future PCRs. UDG is inactivated at high temperature during PCR thermal cycling, thereby allowing amplification of genuine target sequence(s).
	Primer dimers or other primer artifacts are present	Use melting curve analysis of the PCR product to identify primer dimers by their lower melting point temperature. Confirm that your primer designs have low scores (0.0-4.0) when generated by the LUX™ Designer to minimize self-annealing. Redesign primers if necessary. If you are redesigning primers, you can first try redesigning only the unlabeled primer to save the cost of the LUX™ primer. Primer contamination or truncated or degraded primers can also lead to artifacts. Check the purity of your primers by gel electrophoresis. If agarose gels are used, we recommend cooling the gels before visualization with intercalating dyes.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
No amplification curve appears on the qPCR graph	There is no PCR product	Run the PCR product on a gel to determine whether PCR worked. Then proceed to the troubleshooting steps below.
No PCR product is evident, either in the qPCR graph or on a gel	The protocol was not followed correctly	Verify that all steps have been followed and the correct reagents, dilutions, volumes, and cycling parameters have been used.
	Template contains inhibitors, nucleases, or proteases, or has otherwise been degraded.	Purify or re-purify your template.
	Primer designs are not optimal	Confirm that you are using the correct primers for your sequence, the primer design scores are within the 0.0-4.0 range in the LUX™ Designer, and the optimal melting temperatures have been specified. Redesign primers if necessary. When redesigning primers, note that you can first try redesigning only the unlabeled primer to save the cost of the LUX™ primer.
PCR product is evident in the gel, but not on the qPCR graph	qPCR instrument settings are incorrect	Confirm that you are using the correct instrument settings (dye selection, reference dye, filters, acquisition points, etc.).
	Problems with your specific qPCR instrument	For instrument-specific tips and troubleshooting using LUX™ Primers, see the instrument protocols at www.invitrogen.com/lux.
PCR efficiency is above 110%	Template contains inhibitors, nucleases, or proteases, or has otherwise been degraded.	Purify or re-purify your template. Inhibitors in the template may result in changes in PCR efficiency between dilutions
PCR efficiency is below 90%	The PCR conditions are suboptimal	Verify that the amount of primers you are using is correct and that the labeled primer has not been exposed to direct light. Verify that the reagents you are using have not been freeze-thawed multiple times and have not sat at room temperature for too long.
	Reagent concentration in a multiplex reaction may be limiting the rate of the reaction.	Perform a single-plex reaction using the same primers and template to check efficiency. Then determine which one of your primer sets should be in limiting concentration. See the multiplex guidelines on page 11.

Accessory Products

Products

The following products are available for use with LUX™ Primers in real-time qPCR and qRT-PCR protocols:

Product	Amount	Catalog no.
Platinum® Quantitative PCR SuperMix-UDG	100 rxns 500 rxns	11730-017 11730-025
SuperScript™ III Platinum® Two-Step qRT-PCR Kit	100 PCRs 500 PCRs	11734-050 11734-068
SuperScript™ III Platinum® One-Step Quantitative RT-PCR System	100 rxns 500 rxns	11732-020 11732-088
Platinum® *Taq* DNA Polymerase	100 rxns 250 rxns 500 rxns 5,000 rxns	10966-018 10966-026 10966-034 10966-083
Micro-to-Midi Total RNA Purification System	50 rxns	12183-018
TRIzol® Reagent	100 ml 200 ml	15596-026 15596-018
Micro-FastTrack™ 2.0 mRNA Isolation Kit	20 rxns	K1520-02
ROX Reference Dye	500 µl	12223-023
DNase I, Amplification Grade (1 U/µl)	100 U	18068-015
RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 U/µl)	5,000 U	10777-019
10 mM dNTP Mix	100 µl	18427-013
DEPC-treated water	4 x 1.25 ml	10813-012

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