invitrogen™
by life technologies™

CellsDirect™ One-Step qRT-PCR Kits

For one-step real-time quantitative RT-PCR from cell lysate

Catalog Nos. 11753-100, 11753-500, 11754-100, 11754-500

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User Manual

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Kit Contents and Storage

Shipping and Storage

Kit components are shipped on dry ice and should be stored at -20°C. Stability can be extended by storing components at -80°C. Note the following special storage conditions:

Catalog nos. 11753-100 and 11753-500: Store tube of ROX Reference dye at -20°C in the dark.

Catalog nos. 11754-100 and 11754-500: Store the 2X Reaction Mix containing ROX Reference dye at -20°C in the dark.

Kit Components—Catalog nos. 11753-100 and 11753-500

Catalog nos. 11753-100 and 11753-500 contain a separate tube of ROX Reference Dye.

	Kit Size	
Component	**100 rxns**	**500 rxns**
Resuspension Buffer	10 ml	10 ml
Lysis Enhancer	1 ml	1 ml
DNase I, Amplification Grade (1 U/µl)	500 µl	2 × 1.25 ml
10X DNase I Buffer	160 µl	800 µl
25 mM EDTA	400 µl	2 × 1 ml
SuperScript® III RT/Platinum® *Taq* Mix (with RNaseOUT™ Ribonuclease Inhibitor)	100 µl	500 µl
2X Reaction Mix	2 × 1.25 ml	12.5 ml
50 mM $MgSO_4$	1 ml	1 ml
DEPC-treated water	2 ml	12.5 ml
HeLa Total RNA (10 ng/µl)	10 µl	10 µl
ROX Reference Dye	100 µl	500 µl

Kit Components—Catalog nos. 11754-100 and 11754-500

Catalog nos. 11754-100 and 11754-500 contain ROX Reference Dye in the 2X Reaction Mix.

	Kit Size	
Component	**100 rxns**	**500 rxns**
Resuspension Buffer	10 ml	10 ml
Lysis Enhancer	1 ml	1 ml
DNase I, Amplification Grade (1 U/µl)	500 µl	2 × 1.25 ml
10X DNase I Buffer	160 µl	800 µl
25 mM EDTA	400 µl	2 × 1 ml
SuperScript® III RT/Platinum® *Taq* Mix (with RNaseOUT™ Ribonuclease Inhibitor)	100 µl	500 µl
2X Reaction Mix with ROX	2 × 1.25 ml	12.5 ml
50 mM $MgSO_4$	1 ml	1 ml
DEPC-treated water	2 ml	12.5 ml
HeLa Total RNA (10 ng/µl)	10 µl	10 µl

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Additional Products

Product	Size	Catalog No.
LUX™ Custom Primers	To order, visit www.invitrogen.com	
CellsDirect™ Two-Step qRT-PCR Kit	100 rxns 500 rxns	11737-030 11737-038
CellsDirect™ SYBR® Green Two-Step qRT-PCR Kit	100 rxns 500 rxns	11738-060 11738-068
CellsDirect™ cDNA Synthesis System	25 rxns 100 rxns	18080-200 18080-300
CellsDirect™ Resuspension and Lysis Buffer	10 ml Resuspension Buffer and 1 ml Lysis Enhancer	11739-010
Platinum® *Taq* DNA Polymerase	100 rxns 250 rxns 500 rxns	10966-018 10966-020 10966-034

Introduction

System Overview

The CellsDirect™ One-Step qRT-PCR Kit is an optimized kit for the detection and quantification of RNA or DNA directly from mammalian cell lysate, without a separate purification step.

In traditional real-time quantitative RT-PCR (qRT-PCR), you first isolate RNA from cells in a time-consuming procedure that can lead to loss of material. Using the CellsDirect™ One-Step qRT-PCR Kit, you lyse the cells and add the complete lysate directly to a one-step qRT-PCR reaction with minimal handling and sample loss.

You can add DNase I to the lysate as an optional step, to eliminate genomic DNA prior to qRT-PCR. Alternatively, you can analyze genomic DNA targets by omitting the DNase I step and using qPCR primers designed for your genomic sequence of interest.

The CellsDirect™ One-Step qRT-PCR Kit has been optimized for small cell samples, ranging from 10,000 cells down to a single cell. This kit is compatible with fluorogenic primer technology such as LUX™ Primers or fluorogenic probe-based technology such as TaqMan® probes. The kit is also compatible with high-throughput applications and frozen samples obtained by Laser Capture Microdissection (LCM).

Advantages of the Kit

This kit offers the following advantages:

- Eliminates time-consuming purification procedures that contribute to sample loss and PCR inhibition
- Compatible with a wide range of mammalian cell types grown under different treatment conditions
- Total lysate volume may be used (depending on the capacity of your real-time instrument), increasing sensitivity and allowing for detection of rare transcripts
- Convenient one-step enzyme mix includes both SuperScript® III Reverse Transcriptase and Platinum® *Taq* DNA Polymerase
- Compatible with high-throughput applications and frozen samples obtained by LCM
- Can also be used to detect genomic DNA targets

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SuperScript® III RT

SuperScript® III Reverse Transcriptase (RT) is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability (Gerard *et al.*, 1986; Kotewicz *et al.*, 1985). The enzyme can synthesize cDNA at a temperature range of 42-60°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases.

Because SuperScript® III RT is not inhibited significantly by ribosomal and transfer RNA, it can effectively synthesize cDNA directly from total RNA.

Platinum® *Taq* DNA Polymerase

Platinum® *Taq* DNA is a recombinant *Taq* DNA polymerase complexed with a proprietary antibody that inhibits polymerase activity at ambient temperatures (Chou *et al.*, 1992; Sharkey *et al.*, 1994; Westfall *et al.*, 1997). Full polymerase activity is restored after the denaturation step in PCR, providing an automatic "hot start" for increased amplification efficiency, sensitivity, and yield.

Control RNA

HeLa Total RNA is included in the kit for use as a positive control. The concentration of HeLa Total RNA provided (10 ng/µl) is equivalent to 1,000 cells.

Methods

General Guidelines for Lysing Cells

Introduction

This section provides general guidelines for lysing cells.

Note

You may perform an optional DNase I digestion to remove genomic DNA from the lysate prior to qRT-PCR (see page 12). However, if your gene-specific primers are well-designed (*i.e.*, spanning an exon-intron-exon junction to avoid amplifying genomic DNA), you may skip this step for convenience.

Alternatively, you may use this kit to detect genomic DNA targets in the lysate (omitting DNase I digestion).

Cell Types and Density

The SuperScript® One-Step qRT-PCR Kit has been optimized for small cell samples, ranging from 1 to 10,000 cells. This kit is compatible with several different mammalian cell lines, including HeLa, COS-7, 293, Jurkat, CV1, and primary cells, including stem cells and neural cells. Cells may be grown under a variety of conditions and treatments, and any type of culture vessel can be used. This kit is not intended for whole blood or macrophages.

Important

- We recommend using a maximum of 10,000 cells per reaction. Higher numbers of cells may inhibit one-step qRT-PCR and result in reduced yields and/or truncated product.
- Make sure that all solutions and equipment that come in contact with the cells are sterile. Always use proper sterile technique and work in a laminar flow hood when handling cells.

Lysing Larger Volumes of Cells

Introduction

This section provides a protocol for lysing larger volumes of cells—*i.e.*, cells in larger plates and flasks. For smaller samples in tissue-culture wells, see **Lysing Cells in Tissue-Culture Wells**, starting on page 7.

Required Materials

The following materials are provided by the user:

- Mammalian cell cultures in growth medium
- Coulter Counter or hemacytometer
- Centrifuge (for pelleting cells)
- Incubator or thermal cycler preheated to 75°C
- Trypsin (for adherent cultures)
- 1X cold phosphate-buffered saline (PBS), without calcium or magnesium
- 0.2-ml thin-walled PCR tubes or PCR plates
- Ice
- Pipettes

The following materials are provided in the kit:

- Resuspension Buffer
- Lysis Enhancer
- $MgSO_4$, 50 mM (optional)

Note

All steps should be performed on ice, and reagents should be chilled and/or thawed immediately prior to use. The incubator should be **preheated** to 75°C.

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Lysis Procedure

For adherent cell cultures, follow all the steps below. **For cells in suspension, skip Steps 1–5 and proceed to Step 6 below.**

1. Aspirate the media in each dish and wash each dish with an appropriate volume of 1X cold PBS (*e.g.*, for a 10-cm dish or a T75 flask, use 10 ml PBS). Aspirate the PBS.
2. Add enough trypsin to cover the adherent cells in your tissue culture dish, plate, or flask (*e.g.*, for a 10-cm dish, use ~1 ml; for a T75 flask, use ~3 ml).
3. Incubate for 5 minutes on ice or at room temperature.
4. Check for cell detachment under a microscope. If cells have not detached, gently tap the disk or flask to dislodge the cells, or let the cells incubate longer, checking them every minute under a microscope.
5. When all the cells have detached, add serum-containing medium to a final volume equaling the volume of PBS used in the Step 1 (for 6- and 12-well plates, add a 1X–2X volume of medium). Note that the medium must contain serum to inactivate the trypsin.
6. Pipet the cells gently up and down to mix, and then transfer the cell suspension to a centrifuge tube.
7. Spin the cells at 200 × *g* for 5 minutes to pellet (or spin at the recommended speed and time appropriate for your cell line).
8. Aspirate the medium and wash the cell pellet with 5–10 ml of 1X cold PBS.
9. Spin the cells at 200 × *g* for 5 minutes to pellet.
10. Aspirate the PBS and resuspend the pellet in 500 µl to 1 ml of 1X cold PBS. Mix the cell solution gently.

Protocol continued on next page

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Lysis Procedure, continued

Protocol continued from previous page

11. Collect a small aliquot (~10 µl) to verify that the cells are at the desired concentration. Determine cell density electronically using a Coulter Counter or manually using a hemacytometer chamber.
12. The cell density should be <10,000 cells/µl. If necessary, adjust the density using cold PBS. Count the cells again to verify cell concentration.
13. To a 0.2-ml thin-walled PCR tube or plate well **on ice**, add 1 µl of Lysis Enhancer and 10 µl of Resuspension Buffer. **Note:** A Master Mix of Lysis Solution (Lysis Enhancer and Resuspension Buffer) may be prepared for multiple reactions.
14. Transfer 1–2 µl of cells (<10,000 cells) to the PCR tube/well and cap or seal. Vortex briefly and spin down the contents.

    Note: This mixture may be frozen and stored at –80ºC until use. Thaw on ice before proceeding to the next step.
15. Transfer the tube/plate to an incubator, water bath, or thermal cycler that has been preheated to 75ºC and incubate for 10 minutes.
16. After incubation, spin the tube or plate briefly to collect any condensation.
17. Proceed to **DNase I Digestion (Optional),** page 12

    or

    If you do not perform the optional DNase I digestion, adjust the Mg^{2+} concentration by adding 1 µl of 50-mM $MgSO_4$ to each tube/well. Then proceed to **One-Step qRT-PCR Guidelines and Recommendations**, page 13.

Lysing Cells in Tissue-Culture Wells

Introduction

This section provides a protocol for lysing cells directly in tissue-culture wells (*e.g.*, in 6-well to 384-well plates). For cells in larger vessels, see **Lysing Larger Volumes of Cells**, page 4.

Cell Seeding Density

For adherent cells grown in tissue-culture wells, seed cells so that 10 µl of resuspended cells will yield the desired concentration.

Required Materials

The following materials are provided by the user:

- Mammalian cell cultures in growth medium
- Centrifuge (for pelleting cells)
- Incubator or thermal cycler preheated to 75°C
- 1X cold phosphate-buffered saline (PBS), without calcium or magnesium
- 0.2-ml thin-walled PCR tubes or PCR plates
- Ice
- Pipettes

The following materials are provided in the kit:

- Resuspension Buffer
- Lysis Enhancer
- $MgSO_4$, 50 mM (optional)

Preparing Master Mix of Lysis Solution

For multiple reactions, prepare a Master Mix of Lysis Solution (Resuspension Buffer: Lysis Enhancer in a ratio of 10:1). See the chart below for the minimum volume of Lysis Solution required to cover the cells in each type of tissue-culture well. Larger volumes may be used, if desired.

Tissue Culture Plate	Volume per Well		
	Resuspension Buffer	Lysis Enhancer	Total
6 well	400 µl	40 µl	440 µl
12 well	200 µl	20 µl	220 µl
24 well	100 µl	10 µl	110 µl
48 well	50 µl	5 µl	55 µl
96 well	20 µl	2 µl	22 µl
384 well	10 µl	1 µl	11 µl

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Important

If you are processing many samples, additional Resuspension Buffer and Lysis Enhancer may be required. You can order additional CellsDirect Resuspension Buffer and Lysis Enhancer from Invitrogen (see page vi).

Direct Lysis of Cells in Tissue-Culture Wells

For adherent cells grown in tissue-culture wells, perform the following lysis procedure.

1. Aspirate the medium in each well and wash each well with 1X cold PBS without magnesium/calcium. Aspirate the PBS.
2. Add the Lysis Solution master mix (see table on previous page) to each well. The master mix should cover the cells in the well.
3. Incubate the plates on ice for up to 10 minutes. During that period, tap the plate periodically and check the cells under a microscope every 2–3 minutes to see whether they have detached or burst.
4. After 10 minutes, gently pipet the cells up and down to dislodge the remaining attached cells.
5. Transfer 10 µl of the cell suspension to a 0.2-ml thin-walled PCR tube or plate well.
6. Cap or seal the tube/plate and transfer to an incubator or thermal cycler that has been preheated to 75°C. Incubate for 10 minutes.
7. After incubation, spin the tube or plate briefly to collect any condensation.
8. Proceed to **DNase I Digestion (Optional),** page 12

   or

   If you do not perform the optional DNase I digestion, adjust the Mg^{2+} concentration by adding 1 µl of 50-mM $MgSO_4$ to each tube/well. Then proceed to **One-Step qRT-PCR Guidelines and Recommendations**, page 13.

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Lysing Cells Obtained by LCM

Introduction

This section provides a protocol for lysing cells from frozen samples obtained by LCM.

LCM Using Arcturus CapSure® Caps

The protocols in this section assume that you are using frozen LCM samples collected on Arcturus CapSure® caps. Two alternate lysis methods are described: the cap method or the polymer peel method (Gallup *et al.*, 2005).

Required Materials

The following materials are provided by the user:

- Mammalian cells obtained via LCM and immobilized on polymer film lining an Arcturus CapSure® cap
- Centrifuge (for pelleting cells)
- Heat block preheated to 75°C (for the cap method)
- 1.7-ml Eppendorf microcentrifuge tube (for the cap method)
- Thermal cycler or incubator preheated to 50°C and 75°C (for the polymer peel method)
- RNase- and DNase-free forceps (for the polymer peel method)
- 0.2-ml thin-walled PCR tubes
- Pipettes

The following materials are provided in the kit:

- Resuspension Buffer
- Lysis Enhancer
- $MgSO_4$, 50 mM (optional)

Note

For collection of very small samples using LCM (*i.e.*, 100 cells or less), glycogen is commonly suggested. This kit is compatible with samples collected with glycogen.

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Cap Lysis Method

The following method lyses cells obtained by LCM directly on the Arcturus CapSure® cap.

1. For a single reaction, prepare 11 µl of Lysis Solution by adding 1 µl of Lysis Enhancer to 10 µl of Resuspension Buffer. **Note:** A Master Mix may be prepared for multiple reactions.
2. Invert the Arcturus CapSure® cap and carefully pipet 11 µl of Lysis Solution onto the immobilized cells. Be careful to place the drop directly on the sample; surface tension should keep this volume as a drop over the sample.
3. Carefully fit a microcentrifuge tube upside-down onto the cap. Be careful not to disturb the surface tension of the Lysis Solution over the cell sample.
4. With the tube still inverted on the cap, place the cap-and-tube assembly directly on a heat block pre-heated to 75°C and incubate for 15 minutes.
5. After incubation, vortex the inverted cap-and-tube assembly briefly and then turn the tube right-side up and spin down briefly to collect the contents to the bottom of the tube. Discard the cap.
6. Proceed to **DNase I Digestion (Optional),** page 12

   or

   If you do not perform the optional DNase I digestion, adjust the Mg^{2+} concentration by adding 1 µl of 50-mM $MgSO_4$ to the tube. Then proceed to **One-Step qRT-PCR Guidelines and Recommendations**, page 13.

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Polymer Peel Lysis Method

The following method involves peeling off the polymer film with the attached cells from the Arcturus CapSure® cap and then lysing the cells in a tube.

1. Using clean forceps that are RNase and DNase-free, carefully peel off the polymer film "sticker" with the attached cells from the cap.
2. Place the polymer film at the bottom of a 0.2-ml thin-walled PCR tube and add 10 µl of Resuspension Buffer and 1 µl of Lysis Enhancer (a Master Mix may be prepared for multiple reactions).
3. Incubate the tube in a thermal cycler or incubator at 50°C for 10 minutes.
4. Vortex the tube and spin down briefly to collect the polymer and other debris at the bottom of the tube.
5. Transfer the aqueous solution to a new 0.2-ml thin-walled PCR tube.
6. Cap the tube and incubate in a thermal cycler preheated to 75°C for 5 minutes.
7. After incubation, spin the tube briefly to collect any condensation.
8. Proceed to **DNase I Digestion (Optional),** page 12

   or

   If you do not perform the optional DNase I digestion, adjust the Mg^{2+} concentration by adding 1 µl of 50-mM $MgSO_4$ to the tube. Then proceed to **One-Step qRT-PCR Guidelines and Recommendations**, page 13.

DNase I Digestion (Optional)

Introduction

In this optional step, you treat the cell lysate with DNase I to degrade the genomic DNA.

Materials Needed

The following materials are provided in the kit:

- 10X DNase I Buffer
- DNase I, Amplification Grade
- 25 mM EDTA

DNase I Digestion

1. To each tube/plate well, add the following components **on ice**:

Component	Amount per sample
DNase I, Amplification Grade (1 U/µl)	5 µl
10X DNase I Buffer	1.6 µl

2. Mix by gently pipetting up and down or briefly vortexing, and spin briefly to collect the contents.
3. Incubate the tube/plate at 25°C (or room temperature) for 5 minutes. **Note:** A longer incubation time (up to 10 minutes) may be used for larger samples (>5,000 cells). However, incubation times exceeding 10 minutes can greatly reduce yield.
4. Spin briefly, and add 4 µl of 25 mM EDTA to each tube/plate well on ice. Mix by gently pipetting up and down, and spin briefly to collect the contents.
5. Incubate at 70°C for 10 minutes.
6. Spin briefly and place the tube or plate on ice before proceeding to the next section.

Guidelines and Recommendations—One-Step qRT-PCR

Introduction

This section provides guidelines for setting up your one-step qRT-PCR reaction.

Important

Since PCR is a powerful technique capable of amplifying trace amounts of DNA, all appropriate precautions should be taken to avoid cross-contamination.

Amount of Starting Material

The amount of lysate you can use in the qRT-PCR reaction depends on your real-time instrument and the total reaction volume it can accommodate. For a 50-µl total reaction volume, up to 20 µl of lysate may be used. For a 20-µl total reaction volume, up to 10 µl of lysate may be used.

Instrument Compatibility

Catalog nos. 11753-100 and 11753-500:

These kits can be used with a variety of real-time instruments, including but not limited to the ABI PRISM® 7000, 7700, and 7900HT; the ABI 7300 and 7500 Real-Time PCR Systems; the ABI GeneAmp® 5700; the Bio-Rad iCycler™; the Stratagene Mx3000P®, Mx3005P™, and Mx4000®; the Corbett Research Rotor-Gene™; the MJ Research DNA Engine Opticon™, Opticon® 2, and Chromo 4™ Real-Time Detector; and the Cepheid Smart Cycler®.

Optimal cycling conditions will vary with different instruments.

Catalog nos. 11754-100 and 11754-500:

These kits include ROX Reference Dye in the 2X Reaction Mix at a final concentration of 500 nM. This concentration is compatible with the ABI PRISM® 7000, 7700, and 7900HT; the ABI 7300 Real-Time PCR System; and the ABI GeneAmp® 5700.

These kits are *not* compatible with instruments that do not use ROX, or instruments that use ROX at a final concentration lower than 500 nM (*e.g.*, the ABI 7500 and the Stratagene Mx3000P®, Mx3005P™, and Mx4000®).

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Guidelines and Recommendations—One-Step qRT-PCR, continued

Primers

Gene-specific primers are required for one-step qRT-PCR. LUX™ Fluorogenic Primers are available separately from Invitrogen; see below for more information.

A final concentration of 200 nM per primer is effective for most reactions. Doubling the amount of reverse primer (to 400 nM) may improve performance of certain reactions. Optimal results may require a primer titration between 100 and 500 nM.

Detection Methods

LUX™ Fluorogenic Primers

LUX™ Primers are fluorogenic primers for qRT-PCR. Each LUX™ Primer set includes one primer labeled with single fluorophore and one corresponding unlabeled primer. The labeled primer is designed with a hairpin structure that provides built-in fluorescence quenching. When the primer is incorporated into double-stranded PCR product and extended, fluorescence increases by up to 10-fold.

For more information, visit www.invitrogen.com/lux. To design LUX™ Primers for specific targets, visit www.invitrogen.com/dluxdesigner. Predesigned and functionally validated Certified LUX™ Primer Sets are also available.

Dual-Labeled Probes

Fluorescent dual-labeled probe technology such as TaqMan® probes requires two gene-specific primers as well as a probe that hybridizes to the internal portion of the amplicon. The probe sequence should be free of secondary structure and should not hybridize to itself or to primer 3´ ends. The optimal concentration of probe may vary between 50 and 500 nM, with a recommended starting concentration of 100 nM.

Fluorescent Dyes

This kit has been developed and optimized for use with fluorogenic primer or probe-based qPCR detection technology. For a CellsDirect™ kit with fluorescent binding dye technology, we recommend the CellsDirect™ SYBR® Green Two-Step qRT-PCR Kit (see page vi for ordering information).

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Detecting Genomic DNA

To detect genomic DNA targets in the lysate, use primers specific for your targets in the one-step reaction, and omit the 50°C cDNA synthesis step in the cycling program. SuperScript® III RT in the enzyme mix will be denatured during the 95ºC PCR incubation.

Alternatively, you can use 2 units of Platinum® *Taq* DNA Polymerase in place of the SuperScript® III RT/Platinum® *Taq* enzyme mix in the one-step qRT-PCR reaction.

RNaseOUT™

RNaseOUT™ Recombinant Ribonuclease Inhibitor (Cat. No. 10777-019) is included in the SuperScript® III RT/Platinum® *Taq* Mix to safeguard against degradation of target RNA due to ribonuclease contamination.

2X Reaction Mix

2X Reaction Mix consists of a proprietary buffer system, $MgSO_4$, dNTPs, and stabilizers, all at optimized concentrations. Catalog nos. 11754-100 and 11754-500 also include ROX Reference Dye in the 2X Reaction Mix.

Note

Be careful to thaw the 2X Reaction Mix completely before use, and vortex briefly to mix. Incomplete thawing may result in a salt concentration that is too low, which may reduce the efficiency of the qRT-PCR reaction.

Magnesium Concentration

The 2X Reaction Mix provided with each kit supplies a final magnesium concentration of 3 mM. This works well for most targets; however, the optimal concentration may range from 3 to 6 mM. If necessary, use the separate tube of 50-mM magnesium sulfate to increase the magnesium concentration. Use the following table to determine the amount of $MgSO_4$ to add to achieve the specified concentration (in a 50-µl PCR with 25 µl of 2X Reaction Mix):

Volume of 50-mM $MgSO_4$ (per 50-µl Rxn)	Final $MgSO_4$ Conc.
1 µl	4.0 mM
2 µl	5.0 mM
3 µl	6.0 mM

Decrease the amount of water in the reaction accordingly.

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Guidelines and Recommendations—One-Step qRT-PCR, continued

ROX Reference Dye

ROX Reference Dye is used to adjust for non-PCR related fluctuations in fluorescence between qPCR reactions, and provides a stable baseline in multiplex reactions. It is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester.

ROX is either included in the kit in a separate tube (Catalog nos. 11753-100 and 11753-500) or as a component of the 2X Reaction Mix at a final concentration of 500 nM (Catalog nos. 11754-100 and 11754-500).

Melting Curve Analysis

Melting curve analysis (available with LUX™ Fluorogenic Primers) should be performed immediately after qRT-PCR to identify the presence of primer dimers and analyze the specificity of the reaction. Melting curve analysis can identify primer dimers by their lower annealing temperature compared to that of the amplicon. The presence of primer dimers in samples containing template decreases PCR efficiency and obscures analysis and determination of cycle thresholds.

Multiplexing

For multiplex applications, different fluorescent reporter dyes are used to label separate primers or probes for quantification of different genes. For relative expression studies using multiplex PCR, the amount of primer for the reference gene (*e.g.*, β-actin or GAPDH) should be limited to avoid competition between amplification of the reference RNA and the sample gene. In general, the final concentration of the reference gene primer should be between 25 and 100 nM. A primer titration is recommended for optimal results.

If additional optimization is required, we first recommend increasing the $MgSO_4$ in the reaction from 3 mM to 6 mM (using the extra $MgSO_4$ provided in the kit). Then we recommend doubling the amount of Platinum® *Taq* DNA Polymerase to 0.06 U per µl of reaction volume, or 3 U per 50-µl reaction. Add Platinum® *Taq* DNA polymerase stand-alone enzyme (see page vi for ordering information) to double the amount of enzyme.

Cycling Programs—One-Step qRT-PCR

Introduction

This section provides general cycling programs for one-step qRT-PCR on ABI real-time instruments. For more instrument-specific programs, go to www.invitrogen.com/qpcr.

Note

To detect genomic DNA targets, omit the 50°C cDNA synthesis step. SuperScript® III RT will be denatured in the 2-minute 95ºC PCR incubation.

Standard Cycling Program

Program your real-time instrument to perform cDNA synthesis immediately followed by PCR amplification, as shown below. Optimal temperatures and incubation times may vary for different target sequences.

50°C for 15 minutes hold (cDNA synthesis temperature may range from 42–60°C; time may range from 5–20 minutes)

95°C for 2 minutes hold

40–50 cycles of:

- 95°C, 15 seconds
- 60°C, 30–45 seconds (60 seconds for the 7900HT)

For LUX™ Primers only: Perform melting curve analysis. Refer to your specific instrument documentation.

Fast Cycling Program (for the ABI 7500 in Fast Mode)

Program the ABI 7500 with Fast Mode capability to perform cDNA synthesis immediately followed by PCR amplification, as shown below. Optimal temperatures and incubation times may vary for different target sequences.

Select *Fast Mode* on the Thermal Profile tab

50°C for 5 minutes hold

95°C for 2 minutes hold

40–50 cycles of:

- 95°C, 3 seconds
- 60°C, 30 seconds

For LUX™ Primers only: Perform melting curve analysis. Refer to your specific instrument documentation.

One-Step qRT-PCR with Optional ROX

Introduction

This section provides general reaction setup and protocol instructions for kits with ROX supplied as a separate tube (Catalog nos. 11753-100 and 11753-500).

Note

The use of ROX Reference Dye is optional. The following instruments do not use ROX: Bio-Rad iCycler™; Corbett Research Rotor-Gene™; MJ Research DNA Engine Opticon™, Opticon® 2, and Chromo 4™ Real-Time Detector; and Cepheid Smart Cycler®.

Amount of ROX to Use

ROX Reference Dye is supplied at a 25 µM concentration. Use the following table to determine the amount of ROX to use with your particular instrument:

Instrument	Amount of ROX per 50-µl reaction	Final ROX Concentration
ABI 7000, 7300 7700, and 7900HT	1.0 µl	500 nM
ABI 7500; Stratagene Mx3000™, Mx3005P™, and Mx4000™	0.1 µl*	50 nM
*To accurately pipet 0.1 µl per reaction, we recommend diluting ROX 1:10 immediately before use and use 1 µl of the dilution.		

Control Reactions

For the reactions in this section, set up the following controls:

- To test for genomic DNA contamination, prepare a negative-RT control reaction containing 2 units of Platinum® *Taq* DNA Polymerase (see page vi for ordering information) instead of the SuperScript® III RT/Platinum® *Taq* Mix.
- For the positive control, use 1 µl of the control HeLa RNA provided with the kit instead of cell lysate. For the no-template control, omit the lysate.

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Protocol for LUX™ Primers

Use the following protocol with LUX™ Primers. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (*e.g.*, scaled down to a 20-µl reaction volume for 384-well plates). For smaller reactions, note that using a full 1 µl of SuperScript® III RT/Platinum® *Taq* Mix may increase sensitivity.

1. Program your real-time instrument to perform cDNA synthesis immediately followed by PCR amplification, as described on page 17.
2. Set up reactions **on ice**. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (*e.g.*, lysate). Preparation of a master mix is crucial in qRT-PCR to reduce pipetting errors.

   Note: Be careful to thaw the 2X Reaction Mix completely before use and vortex to mix.

Component	Single rxn
SuperScript® III RT/Platinum® *Taq* Mix	1 µl
2X Reaction Mix	25 µl
LUX™ labeled primer, 10 µM	1 µl
Unlabeled primer, 10 µM	1 µl
ROX Reference Dye (optional)	1 µl/0.1 µl *
Lysate	2–20 µl
DEPC-treated water	to 50 µl

   *See the table on page 18 for the amount/concentration of ROX to use for your specific instrument.
3. Cap or seal the reaction tube/PCR plate. Centrifuge briefly to make sure that all components are at the bottom of the tube/plate.
4. Place reactions in a preheated real-time instrument programmed as described in step 1. Collect data and analyze results.

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Protocol for TaqMan® Probes

Use the following protocol with TaqMan® Probes. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (*e.g.,* scaled down to a 20-µl reaction volume for 384-well plates). For smaller reactions, note that using a full 1 µl of SuperScript® III RT/Platinum® *Taq* Mix may increase sensitivity.

1. Program your real-time instrument to perform cDNA synthesis immediately followed by PCR amplification, as described on page 17.
2. Set up reactions **on ice**. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (*e.g.,* lysate). Preparation of a master mix is crucial in qRT-PCR to reduce pipetting errors.

   Note: Be careful to thaw the 2X Reaction Mix completely before use and vortex to mix.

Component	Single rxn
SuperScript® III RT/Platinum® *Taq* Mix	1 µl
2X Reaction Mix	25 µl
Forward primer, 10 µM	1 µl
Reverse primer, 10 µM	1 µl
Fluorogenic probe	1 µl
ROX Reference Dye (optional)	1 µl/0.1 µl *
Lysate	2–20 µl
DEPC-treated water	to 50 µl

   *See the table on page 18 for the amount/concentration of ROX to use for your specific instrument.

3. Cap or seal the reaction tube/PCR plate. Centrifuge briefly to make sure that all components are at the bottom of the tube/plate.
4. Place reactions in a preheated real-time instrument programmed as described in step 1. Collect data and analyze results.

One-Step qRT-PCR with ROX in the 2X Reaction Mix

Introduction

This section provides general reaction setup and protocol instructions for kits with ROX included in the 2X Reaction Mix (Catalog nos. 11754-100 and 11754-500).

Note

These kits include ROX Reference Dye in the 2X Reaction Mix at a final concentration of 500 nM. For information about instrument compatibility, see page 13.

Control Reactions

For the reactions in this section, set up the following controls:

- To test for genomic DNA contamination, prepare a negative-RT control reaction containing 2 units of Platinum® *Taq* DNA Polymerase (see page vi for ordering information) instead of the SuperScript® III RT/Platinum® *Taq* Mix.
- For the positive control, use 1 µl of the control HeLa RNA provided with the kit instead of cell lysate. For the no-template control, omit the lysate.

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Protocol for LUX™ Primers

Use the following protocol with LUX™ Primers. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (*e.g.*, scaled down to a 20-µl reaction volume for 384-well plates). For smaller reactions, note that using a full 1 µl of SuperScript® III RT/Platinum® *Taq* Mix may increase sensitivity.

1. Program your real-time instrument to perform cDNA synthesis immediately followed by PCR amplification, as described on page 17.
2. Set up reactions **on ice**. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (*e.g.*, lysate). **Note:** Preparation of a master mix is *crucial* in qRT-PCR to reduce pipetting errors.

Component	Single rxn
SuperScript® III RT/Platinum® *Taq* Mix	1 µl
2X Reaction Mix with ROX	25 µl
LUX™ labeled primer, 10 µM	1 µl
Unlabeled primer, 10 µM	1 µl
Lysate	2–20 µl
DEPC-treated water	to 50 µl

3. Cap or seal the reaction tube/PCR plate. Centrifuge briefly to make sure that all components are at the bottom of the tube/plate.
4. Place reactions in a preheated real-time instrument programmed as described in step 1. Collect data and analyze results.

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Protocol for TaqMan® Probes

Use the following protocol with TaqMan® Probes. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (*e.g.,* scaled down to a 20-µl reaction volume for 384-well plates). For smaller reactions, note that using a full 1 µl of SuperScript® III RT/Platinum® *Taq* Mix may increase sensitivity.

1. Program your real-time instrument to perform cDNA synthesis immediately followed by PCR amplification, as described on page 17.
2. Set up reactions **on ice**. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (*e.g.,* lysate). **Note:** Preparation of a master mix is *crucial* in qRT-PCR to reduce pipetting errors.

Component	Single rxn
SuperScript® III RT/Platinum® *Taq* Mix	1 µl
2X Reaction Mix with ROX	25 µl
Forward primer, 10 µM	1 µl
Reverse primer, 10 µM	1 µl
Fluorogenic probe	1 µl
Lysate	2–20 µl
DEPC-treated water	to 50 µl

3. Cap or seal the reaction tube/PCR plate. Centrifuge briefly to make sure that all components are at the bottom of the tube/plate.
4. Place reactions in a preheated real-time instrument programmed as described in step 1. Collect data and analyze results.

Appendix

Troubleshooting

Problem	Possible Cause	Suggested Solution
Cells in tissue-culture wells do not detach/burst	Incubation temperature of lysis reaction is too low	Incubate lysis reaction at room temperature instead of on ice.
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	Procedural error	Confirm that all steps were followed. Use the control RNA to verify the efficiency of the reaction (see the next page on troubleshooting with the Control RNA).
	RNA is degraded	Add control total HeLa RNA to sample to determine if RNase is present in the first-strand reaction. The optional DNase I digestion can hydrolyze the RNA in the sample, if the digestion time is too long. Use a digestion time of <10 minutes. Take appropriate cautions to prevent RNase contamination.
	Fluorescent probe not functional	Validate probe design and presence of fluorophore and quencher: Treat TaqMan® Probe with DNase, and check for increase in fluorescence. Redesign and/or resynthesize probe if necessary.
	Target mRNA contains strong transcriptional pauses	Maintain an elevated temperature after the annealing step. Redesign the primers. Increase the temperature of cDNA synthesis (up to 60°C).
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 protocols, tips, and troubleshooting, including information about LUX™ Primers, visit www.invitrogen.com/qpcr. For additional information about probes, consult your instrument documentation and/or your probe technology documentation.

Continued on next page

Troubleshooting, continued

Problem	Possible Cause	Suggested Solution
Poor sensitivity	Not enough template RNA	Increase the number of cells used
	Scaled-down reaction volume (*e.g.,* 20 µl) does not include enough enzyme	Use 1 µl of SuperScript® III RT/Platinum® *Taq* Mix, even in smaller reaction volumes.
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 if possible, and/or run the PCR products on a 4% agarose gel after the reaction to identify contaminants. Include the optional DNase I digestion step.
	Primer dimers or other primer artifacts are present	Use melting curve analysis to identify primer dimers by their lower melting temperature if possible. We recommend using validated pre-designed primer sets or designing primers or primer/probe combinations using dedicated software programs or primer databases. Check the purity of your primers by gel electrophoresis.
Product detected at higher than expected cycle number	RNA is degraded	Add control total HeLa RNA to sample to determine if RNase is present in the first-strand reaction. The optional DNase I digestion can hydrolyze the RNA in the sample, if the digestion time is too long. Use a digestion time of <10 minutes. Take appropriate cautions to prevent RNase contamination.
	Inefficient cDNA synthesis	Adjust cDNA synthesis temperature and/or primer design. Double the amount of reverse primer (*e.g.,* to 400 nM).
	Inefficient PCR amplification	Optimize PCR conditions: Adjust annealing temperature as necessary. Increase magnesium concentration. Redesign primers.

Continued on next page

Troubleshooting, continued

Product detected at lower-than-expected cycle number, and/or positive signal from no-template controls	Template or PCR carry-over contamination	Isolate source of contamination and replace reagent(s). Use separate dedicated pipettors for reaction assembly and post-PCR analysis. Assemble reactions (except for lysate) in a DNA-free area. Use aerosol-resistant pipet tips or positive displacement pipettors.
Unexpected bands after electrophoretic analysis	Contamination by genomic DNA	Include the optional DNase Digestion step. For larger samples (>1,000 cells), use a longer DNase I incubation time, *i.e.,* up to 10 minutes. Design primers that anneal to the target sequence in exons on both sides of an intron or the exon/exon boundary of mRNA to differentiate between amplification of cDNA and potential contaminating genomic DNA. To test if products were derived from DNA, prepare a negative RT control.
	Nonspecific annealing of qPCR primers	Vary the annealing conditions. Optimize magnesium concentration for each template and primer combination.

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