

SuperScript[®] III Platinum[®] CellsDirect Two-Step qRT- PCR Kit

**For two-step real-time quantitative RT-PCR from
cell lysate**

Catalog Nos. 11737-030 and 11737-038

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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. ROX Reference Dye should be stored in the dark.

Kit Size and Modules Each kit includes a cDNA synthesis module and a qPCR module. Each cDNA synthesis reaction provides enough cDNA for multiple qPCR reactions.

<u>Kit Catalog Number</u>	<u>Number of Reactions</u>	
	<u>cDNA Synthesis</u>	<u>qPCR</u>
11737-030	25	100
11737-038	100	500

cDNA Synthesis Module	<u>Component</u>	<u>100-rxn kit</u>	<u>500-rxn kit</u>
	Resuspension Buffer	2 × 250 µl	2 × 1 ml
	DNase I (1 U/µl)	125 µl	500 µl
	10X DNase I Buffer	40 µl	160 µl
	25-mM EDTA	120 µl	400 µl
	RT Enzyme Mix (contains SuperScript® III RT, 100 units/µl; and RNaseOUT™ Recombinant Ribonuclease Inhibitor, 20 units/µl)	50 µl	200 µl
	2X RT Reaction Mix*	500 µl	2 × 1 ml
	<i>E. Coli</i> RNase H (2 U/µl)	30 µl	100 µl
	HeLa Total RNA (10 ng/µl)	10 µl	10 µl
	Lysis Enhancer	125 µl	500 µl
	*Oligo(dT) ₂₀ (2.5 µM), random hexamers (2.5 ng/µl), 10 mM MgCl ₂ , and dNTPs		

qPCR Module	<u>Component</u>	<u>100-rxn kit</u>	<u>500-rxn kit</u>
	Platinum® Quantitative PCR SuperMix-UDC*	2 × 1.25 ml	12.5 ml
	50-mM Magnesium Chloride	1 ml	2 × 1 ml
	ROX Reference Dye	100 µl	500 µl
	*60 U/ml Platinum® <i>Taq</i> DNA polymerase, 40-mM Tris-HCl (pH 8.4), 100-mM KCl, 6-mM MgCl ₂ , 400-µM dGTP, 400-µM dATP, 400-µM dCTP, 800-µM dUTP, 40 U/ml UDG, and stabilizers.		

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Kit Contents and Storage, continued

**Materials
Supplied by
the User**

The following additional items are required for use with this kit:

- Coulter Counter or hemacytometer
- Microcentrifuge
- qPCR instrument
- Trypsin (for adherent cell cultures only)
- 1X cold phosphate-buffered saline PBS, without calcium or magnesium
- 0.2-ml thin-walled PCR tubes or 96-well PCR plates
- Bovine Serum Albumin (BSA), UltraPure (1 mg/ml)
- Ice
- Pipettes
- Disposable gloves

Introduction

System Overview

The SuperScript® III Platinum® CellsDirect Two-Step qRT-PCR Kit is an optimized kit for synthesizing first-strand cDNA directly from mammalian cell lysate without first isolating RNA, and then amplifying the cDNA in a real-time quantitative PCR (qPCR) reaction using Platinum® Quantitative PCR SuperMix-UDG.

In traditional qRT-PCR, RNA is first isolated from cells in a time-consuming procedure that can lead to a loss of material. Using the CellsDirect cDNA Synthesis System, the cells are lysed and the cDNA is generated from the lysate in a single tube with minimal handling and no sample loss. DNase I is added to eliminate genomic DNA prior to first-strand synthesis. After synthesis, the first-strand cDNA can be transferred directly to the qPCR reaction without intermediate organic extractions or ethanol precipitations.

This kit has been optimized for small cell samples, ranging from 10,000 cells down to a single cell. The use of SuperScript® III Reverse Transcriptase ensures high specificity and high yields of cDNA from small amounts of starting material—as little as 10 pg total RNA. The use of Platinum® Quantitative PCR SuperMix-UDG ensures optimal performance using a variety of qPCR detection technologies, including LUX™ Fluorogenic Primers and fluorescent hybridization probe technologies such as TaqMan® probes and molecular beacons.

Advantages of the Kit

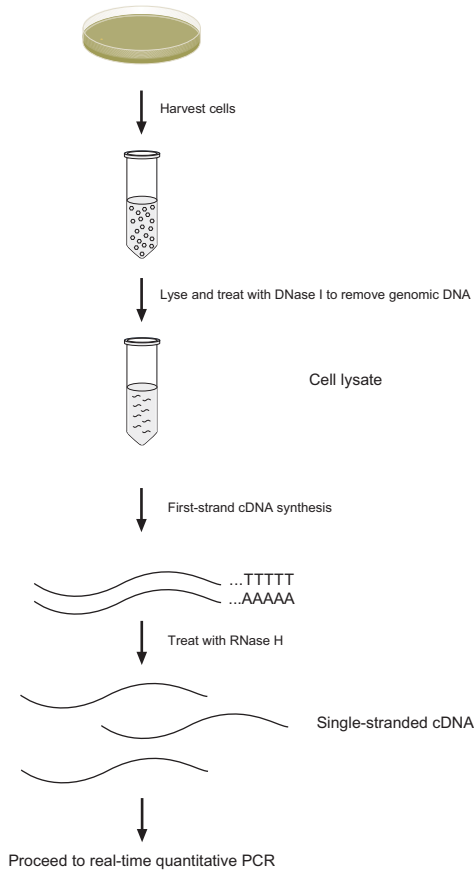
This kit has the following advantages:

- Compatible with a wide range of mammalian cell types grown under different treatment conditions
 - Cell lysis and first-strand cDNA synthesis in the same tube minimizes reagent loss, sample loss, and handling time
 - Total lysate volume is used in the cDNA synthesis reaction, providing greater yields with a limited number of cells and allowing for detection of rare transcripts
 - SuperScript® III Reverse Transcriptase, with reduced RNase H activity and higher thermal stability, produces high yields of cDNA in the first-strand synthesis reaction for greater sensitivity and enhanced detection of rare transcripts
 - Platinum® Quantitative PCR SuperMix-UDG ensures optimal sensitivity and performance using a variety of qPCR detection technologies, with built-in carryover contamination protection and a linear dose response over a wide range of target concentrations
-

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Introduction, Continued

Diagram of cDNA Synthesis from Cell Lysate



SuperScript® III RT

SuperScript® III Reverse Transcriptase is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The enzyme can be used to synthesize first-strand cDNA at temperatures up to 55°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 first-strand cDNA directly from total RNA. The concentration of SuperScript® III RT in this system has been optimized to synthesize first-strand cDNA from total RNA in cell lysate.

Continued on next page

Introduction, Continued

Platinum® Quantitative PCR SuperMix- UDG

Platinum® Quantitative PCR SuperMix-UDG is a ready-to-use reaction cocktail containing all components, except primers, for the amplification and detection of DNA in qPCR. It contains Platinum® *Taq* DNA polymerase, Mg^{++} , uracil DNA glycosylase (UDG), proprietary stabilizers, and deoxyribonucleotide triphosphates, with dUTP instead of dTTP. The concentration of the SuperMix allows for the addition of primers, template, and (if necessary) probes or dyes.

Platinum® *Taq* DNA polymerase is precomplexed with specific monoclonal antibodies that inhibit polymerase activity during reaction assembly at room temperature. Full polymerase activity is restored after the denaturation step in PCR cycling, providing an automatic “hot start” in PCR and thereby increasing amplification efficiency, sensitivity, and yield.

UDG and dUTP are included in the mixture to prevent the reamplification of carryover PCR products between reactions. dUTP in the mix ensures that any amplified DNA will contain uracil. UDG, or uracil-N-glycosylase, removes uracil residues from single- or double-stranded DNA, preventing dU-containing DNA from serving as template in future PCRs. Incubation of subsequent PCRs with UDG before cycling destroys any contaminating dU-containing PCR product from previous reactions. After this decontamination step, UDG is inactivated by the high temperatures during normal PCR cycling, thereby allowing the amplification of genuine target sequence(s).

Control RNA

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

Methods

Lysing Cells

Introduction

In this step, you lyse your cells in Resuspension Buffer and Lysis Enhancer and perform a DNase I digestion to remove genomic DNA from the sample.

Cell Types and Density

This 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 K562. Cells may be grown under a variety of conditions and treatments. Any type of culture vessel can be used.



Important

- We recommend using a maximum of 10,000 cells per reaction. Higher numbers of cells may inhibit reverse transcription and result in reduced yields and/or truncated cDNA 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.

Required Materials

The following materials are provided by the user:

- Mammalian cell cultures in growth media
- Coulter Counter or hemacytometer
- Centrifuge (for pelleting cells)
- Incubator, water bath, or thermal cycler preheated to 75°C
- Trypsin (for adherent cell cultures only)
- 1X cold phosphate-buffered saline (PBS), without calcium or magnesium
- 0.2-ml thin-walled PCR tubes or 96-well PCR plates
- Ice
- Pipettes

The following materials are provided in the kit:

- Resuspension Buffer
- Lysis Enhancer
- DNase I, Amplification Grade (1 U/ μ l)
- 10X DNase I Buffer
- EDTA, 25 mM
- Optional: Control HeLa Total RNA



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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Lysing Cells, continued

Lysing Adherent Cells or Cells in Suspension

Use the following lysis procedure for adherent cell cultures in vessels larger than 24-well plates. For cells in suspension, skip Steps 1–4 and proceed to Step 5 below.

1. 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).
2. Incubate for 5 minutes at room temperature or in a 37°C incubator.
3. Check for cell detachment under a microscope. If cells have not detached, gently tap the dish or flask to dislodge the cells, or let the cells incubate longer, checking them every minute under a microscope.
4. When all the cells have detached, add serum-containing media to a final volume of 10 ml (for 6- and 12-well plates, add a 1X–2X volume of media). Note that the media must contain serum to inactivate the trypsin.
5. Pipet the cells gently up and down to mix, and then transfer the cell suspension to a centrifuge tube.
6. Spin the cells at $200 \times g$ for 5 minutes to pellet (or spin at the recommended speed and time for your cell line).
7. Aspirate the media and wash the cell pellet with 5–10 ml of 1X cold PBS.
8. Spin the cells at $200 \times g$ for 5 minutes to pellet.
9. Aspirate the PBS and resuspend the pellet in 500 μ l to 1 ml of 1X cold PBS. Mix the cell solution gently.
10. Collect a small aliquot to verify that the cells are at the desired concentration. Determine cell density electronically using a Coulter Counter or manually using a hemacytometer chamber.
11. Adjust the cell density using cold PBS so that it falls within the range of 1–10,000 cells/ μ l. Count the cells again to verify cell concentration.
12. To a 0.2-ml thin-walled PCR tube or plate well **on ice**, add 1 μ l of Lysis Enhancer and 10 μ l of Resuspension Buffer. **Note:** A master mix of Lysis Enhancer and Resuspension Buffer may be prepared for multiple reactions.
13. Transfer 1–2 μ l of cells (<10,000 cells) to the PCR tube/well.
Control Reaction: For the control reaction, add 1 μ l of Control HeLa Total RNA to the PCR tube or plate well instead of cell lysate.
14. Transfer the tube/plate to an incubator, water bath, or thermal cycler preheated to 75°C and incubate for 10 minutes.
Control Reaction: For the control reaction, incubate for 3 minutes.
15. After incubation, spin briefly to collect the condensation and proceed to **DNase I Digestion**, page 7.

Continued on next page

Lysing Cells, continued



Note

For adherent cells grown in tissue culture wells, note the following:

- Seed cells in tissue culture wells so that 10 μ l of resuspended cells will yield the desired concentration.
- **Master mix:** Before starting the following procedure, prepare a master mix of Lysis Enhancer and Resuspension buffer for multiple reactions. Add 1 μ l of Lysis Enhancer for every 10 μ l of Resuspension Buffer.



Important

You can order additional CellsDirect Resuspension Buffer and Lysis Enhancer from Invitrogen (Catalog no. 11739-010). Additional buffer and enhancer may be required if you are using 48-well or 24-well plates in your experiments.

Lysing Cells in Tissue Culture Wells

For adherent cells grown in tissue culture wells (i.e., in 24-well, 48-well, or 96-well plates), perform the following lysis procedure.

1. Aspirate the media in each well and wash each well with 1X cold PBS. Aspirate the PBS.
2. Add the Lysis Enhancer/Resuspension Buffer master mix (see **Note** above) to each well. For 96-well plates, add at least 11 μ l of the buffer/enhancer mix to each well. For 24-well plates, add at least 110 μ l of the buffer/enhancer mix 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 μ l of the cell suspension to a 0.2-ml thin-walled PCR tube or plate well.

Control Reaction: For the control reaction, add 10 μ l of Resuspension Buffer and 1 μ l of Lysis Enhancer to a PCR tube or plate well, and then add 1 μ l of Control HeLa Total RNA.

6. Transfer the tube/plate to an incubator or thermal cycler preheated to 75°C and incubate for 10 minutes.

Control Reaction: For the control reaction, incubate for 3 minutes.

7. After incubation, spin briefly to collect the condensation, and proceed to **DNase I Digestion**, page 7.

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Lysing Cells, continued

DNase I Digestion

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

1. Place each tube/plate from Step 15, page 5, or Step 7, page 6, on ice, and add the following:

<u>Component</u>	<u>Amount</u>
DNase I, Amplification Grade (1 U/ μ l)	5 μ l
10X DNase I Buffer	1.6 μ l

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 cDNA yield.
4. Spin briefly, and add 4 μ l of 25-mM EDTA to each tube/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 proceed to **First-Strand cDNA Synthesis**, page 8.

First-Strand cDNA Synthesis

Required Materials

The following materials are provided by the user:

- Thermal cycler preheated to 25°C
- Ice
- Pipettes

The following materials are provided in the kit:

- 2X RT Reaction Mix
- RT Enzyme Mix (contains SuperScript® III RT, 100 units/μl; and RNaseOUT™ Recombinant Ribonuclease Inhibitor, 20 units/μl)
- RNase H (2 U/μl)

First-Strand cDNA Synthesis

1. To each tube/plate from **DNase I Digestion**, Step 6, page 7, add the following:

<u>Component</u>	<u>Amount</u>
2X RT Reaction Mix	20 μl
RT Enzyme Mix*	2 μl

*For negative RT controls, use 1 μl of sterile, distilled water and 1 μl of RNaseOUT™ Recombinant Ribonuclease Inhibitor instead of the RT Enzyme Mix.

2. Spin the tube/plate briefly to collect the contents.
3. Transfer the tube/plate to a thermal cycler preheated to 25°C and incubate for 10 minutes.
4. Incubate at 50°C for 20 minutes.
5. Inactivate the reaction at 85°C for 5 minutes.
6. Add 1 μl of RNase H (2 U/μl) to each tube/well and incubate at 37°C for 20 minutes.
7. Chill the reaction on ice, and store at –20°C or proceed directly to qPCR.

qPCR — Guidelines and Recommendations

Introduction

After first-strand cDNA synthesis, you can proceed directly to qPCR without additional purification.

Required Materials

The following materials are provided by the user:

- qPCR instrument
- Appropriate PCR plates/tubes for instrument
- Primers/probes
- Pipettes

The following materials are provided in the kit:

- Components of the qPCR module
-

Instrument Settings

qPCR can be performed with a variety of instruments, including the ABI PRISM® 7000/7300/7500/7700/7900 and GeneAmp® 5700, Bio-Rad iCycler™, Stratagene Mx4000® and Mx3000P™, Corbett Research Rotor-Gene™, MJ Research DNA Engine Opticon® and Opticon® 2, Cepheid Smart Cycler®, and Roche LightCycler®. Please refer to your instrument user manual for operating instructions. Optimal cycling conditions will vary with different machines.

The protocols on the following pages have been optimized for the ABI PRISM® 7700 and the Roche LightCycler®.

ROX Reference Dye

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. Its use is optional. 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. To prepare a master mix:

1. Add ROX Reference Dye to Platinum® Quantitative PCR SuperMix-UDG, at a ratio of 1 µl of ROX for every 25 µl of SuperMix-UDG.
2. Mix by vortexing for 10 seconds.
3. Store mixture at either -20°C or 4°C in the dark. Use 26 µl of a ROX/SuperMix-UDG mixture per 50 µl of reaction volume.

Note: Use of ROX Reference Dye is not supported on the LightCycler®. The iCycler™ typically uses fluorescein as the reference dye; see the iCycler™ manual for more information. ROX Reference Dye is not required on the ABI PRISM® 7900.

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qPCR — Guidelines and Recommendations, continued

Detection Methods

LUX™ Primers

LUX™ Primers are fluorogenic primers for qPCR and qRT-PCR. Each LUX™ Primer set includes one primer labeled with single fluorophore (FAM or JOE) and one corresponding unlabeled primer. The labeled primer is designed with a hairpin structure that provides built-in fluorescent quenching, enabling detection of your gene of interest without a separate quencher or probe.

To design and order custom LUX™ Primers, visit www.invitrogen.com/LUX. Predesigned and functionally validated Certified LUX™ Primer Sets for Housekeeping Genes are also available from Invitrogen.

Dual-Labeled Probes

Fluorescent dual-labeled probe technology such as TaqMan® probes requires two PCR 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 800 nM, with a recommended starting concentration of 100 nM.

PCR primers used with probes should be designed according to standard PCR guidelines. Optimal results may require a titration of primer concentrations between 100 and 500 nM. A final concentration of 200 nM per primer is effective for most reactions.

Fluorescent Dyes

This kit has been developed and optimized for fluorogenic primer- or probe-based detection technology. We do not recommend using it with fluorescent binding dyes, such as SYBR® Green I.

Melting Curve Analysis

If possible, melting curve analysis should be performed during qPCR 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.

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.

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qPCR — Guidelines and Recommendations, continued

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 MgCl_2 in the reaction from 3 mM to 6 mM (using the extra MgCl_2 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 (Catalog no. 10966-018) to double the amount of enzyme.

Magnesium Concentration

Platinum[®] Quantitative PCR SuperMix-UDG includes magnesium chloride at a final concentration of 3 mM. If necessary, use the 50-mM magnesium chloride solution included in the kit to increase the magnesium concentration to up to 6 mM.

Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) is required in Roche LightCycler[®] reactions because the glass capillaries in the LightCycler[®] have a high surface-to-volume ratio and the glass surface binds molecules like *Taq* DNA polymerase. 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).



Note

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

qPCR — Instruments Using PCR Tubes/Plates

Introduction

This section provides a cycling program, reaction mixture, and protocol for qPCR instruments that use PCR tubes/plates (e.g., ABI PRISM®, Stratagene Mx4000® and Mx3000P™, Corbett Research Rotor-Gene™, MJ Opticon®). For a protocol using the Roche LightCycler®, see page 14.

The tables below summarize the cycling conditions and reaction components for LUX™ Primers and dual-labeled probes. After programming the instrument and preparing the reaction mix, follow the protocol on the following page to perform the reaction.



You can use 1–8 µl of cDNA template in the following protocol, depending on the concentration of the template. Adjust the volume of water in the master mix accordingly for a final reaction volume of 50 µl. We recommend 4 µl of template as a general starting point.

Cycling Program

Program the qPCR instrument as follows:

- 50°C for 2 minutes hold (UDG incubation)
- 95°C for 2 minutes hold
- 50 cycles of:
 - 95°C, 15 seconds
 - 60°C, 30 seconds

Melting Curve Analysis: Refer to instrument documentation. (Melting curve analysis is not available with TaqMan® Probes.)

Master Mix

Use the following table to prepare a master mix of all components except template and water. **Note:** Preparation of a master mix is **crucial** in qPCR to minimize pipetting errors.

Component	1 rxn	50 rxns
Platinum® Quantitative PCR SuperMix-UDG ¹	25 µl	1250 µl
ROX Reference Dye (optional)	1 µl	50 µl
Forward primer, 10 µM	1 µl	50 µl
Reverse primer, 10 µM	1 µl	50 µl
TaqMan® only: Probe, 10 µM	0.5 µl	25 µl
Autoclaved, distilled water ²	to 42–49 µl	to 2100–2550 µl

¹Final concentration: 0.06 U/µl Platinum® Taq DNA polymerase, 20-mM Tris-HCl (pH 8.4), 50-mM KCl, 3-mM MgCl₂, 200-µM dGTP, 200-µM dATP, 200-µM dCTP, 400-µM dUTP, 1 U UDG

²Volume of water used in the master mix will depend on template volume used in the reaction (see steps 3–4 next page).

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qPCR — Instruments Using PCR Tubes/Plates, continued

Protocol

1. Program the qPCR instrument to perform a brief UDG incubation immediately followed by PCR amplification, as shown on the previous page. Optimal cycling temperatures and times may vary for different target sequences, primer sets, and instruments.
 2. Prepare a master mix of all components except template as specified on the previous page.
 3. For each reaction, add 42–49 μl of the master mix (depending on template volume) to a 0.2-ml microcentrifuge tube or each well of a 96-well PCR plate.
 4. Add 1–8 μl of cDNA template from the first-strand synthesis reaction (Step 7, page 8) to each reaction vessel, for a final reaction volume of 50 μl . (Use 4 μl of template as a general starting point.) Cap or seal the tube/plate.
 5. Gently mix and make sure that all components are at the bottom of the reaction vessel. Centrifuge briefly if needed.
 6. Place reactions in a thermal cycler programmed as described above. After cycling, hold the reaction at 4°C until further analysis. Collect and analyze the results.
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Introduction

This section provides a cycling program, reaction mixture, and protocol for the Roche LightCycler®.

The tables below summarize the cycling conditions and reaction components for LUX™ Primers and dual-labeled probes. After programming the instrument and preparing the reaction mix, follow the protocol on the following page to perform the reaction.

Cycling Program

Program the qPCR instrument as follows:

Program choice: Amplification
Analysis mode: Quantification
50°C for 2 minutes hold (UDG incubation)
92°C for 1 minute hold
50 cycles of:
 92°C, 5 seconds
 60°C, 30 seconds (single acquire)

Melting Curve Analysis: Refer to instrument documentation.
(Melting curve analysis is not available with TaqMan® Probes.)

Master Mix

Use the following table to prepare a master mix of all components except template. **Note:** Preparation of a master mix is **crucial** in qPCR to minimize pipetting errors.

<u>Component</u>	<u>1 rxn</u>	<u>34 rxns</u>
Platinum® Quantitative PCR SuperMix-UDG ¹	10 µl	340 µl
BSA, UltraPure (1 mg/ml) ²	1 µl	34 µl
Forward primer, 10 µM	1 µl	34 µl
Reverse primer, 10 µM	1 µl	34 µl
TaqMan® only: Probe, 10 µM	0.5 µl	17 µl
Autoclaved, distilled water	to 18 µl	to 612 µl

¹Final concentration: 0.06 U/µl Platinum® Taq DNA polymerase, 20-mM Tris-HCl (pH 8.4), 50-mM KCl, 3-mM MgCl₂, 200-µM dGTP, 200-µM dATP, 200-µM dCTP, 400-µM dUTP, 1 U UDG

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

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Protocol

1. Program the LightCycler® to perform a brief UDG incubation immediately followed by PCR amplification, as shown on the previous page. Optimal cycling temperatures and times may vary for different target sequences and primer sets.
 2. Set the fluorescence on the LightCycler® to the F1 channel.
 3. Prepare a master mix of all components except template as specified on the previous page.
 4. For each reaction, add 18 µl of the master mix to each capillary tube.
 5. Add 2 µl of the cDNA from the first-strand synthesis reaction (Step 7, page 8) to each capillary tube for a final reaction volume of 20 µl, and cap the tube.
 6. Centrifuge tubes at $700 \times g$ for 5 seconds.
 7. Place reaction tubes in the rotor of the LightCycler® and run the program. After cycling, hold the reaction at 4°C until further analysis. Collect and analyze the results.
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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 first-strand 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. A longer DNase I digestion can hydrolyze the RNA in the sample. Use a digestion time of <10 minutes. Maintain aseptic conditions 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. Increase the temperature of first-strand reaction (up to 55°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 tips and troubleshooting using LUX™ Primers, see the instrument protocols at www.invitrogen.com/lux . For instrument-specific information about probes, consult your instrument documentation and/or your probe technology documentation.
Poor sensitivity	Not enough starting template RNA	Increase the number of cells used
Higher than expected signal	Too much first-strand product was used in qPCR	Decrease amount of the first-strand product in qPCR.

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Troubleshooting, continued

Problem	Possible Cause	Suggested 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 if possible, and/or run the PCR products on a 4% agarose gel after the reaction 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.
	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. 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.
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. A longer DNase I digestion can hydrolyze the RNA in the sample. Use a digestion time of <10 minutes. Maintain aseptic conditions to prevent RNase contamination.
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 target addition) in a DNA-free area. Use aerosol-resistant pipet tips or positive displacement pipettors.
Unexpected bands after electrophoretic analysis	Contamination by genomic DNA	Do not omit the DNase Digestion step on page 7. For larger samples (>1,000 cells), use a longer DNase I incubation time, i.e., up to 10 minutes. Design primers that anneal to sequence in exons on both sides of an intron or exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and products 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.

Related Products

Product	Size	Cat. No.
CellsDirect Resuspension and Lysis Buffer	10 ml Resuspension Buffer and 1 ml Lysis Enhancer	11739-010
E-Gel® Pre-cast Agarose Gels		
0.8% Starter Pak	9 gels and base	G5000-08
1.2% Starter Pak	9 gels and base	G5000-01
2% Starter Pak	9 gels and base	G5000-02
4% Starter Pak	9 gels and base	G5000-04
UltraPure™ Agarose	100 g	15510-019
	500 g	15510-027
UltraPure™ Agarose 1000	100 g	10975-035
100-bp DNA Ladder	50 µg	15628-019
123-bp DNA Ladder	100 µg	15613-011
	250 µg	15613-029
1-Kb Plus DNA Ladder	250 µg	10787-018
	1,000 µg	10787-026

Technical Support

Web Resources



Visit the Invitrogen website at www.invitrogen.com for:

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