invitrogen™
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SuperScript® III Platinum® CellsDirect Two-Step qRT-PCR Kit with SYBR® Green

For two-step real-time quantitative RT-PCR from cell lysate using SYBR® Green I fluorescent dye

Catalog Nos. 11738-060 and 11738-068

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MAN0000472

User Manual

Table of Contents

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.

	Number of Reactions	
Kit Catalog Number	**cDNA Synthesis**	**qPCR**
11737-060	25	100
11737-068	100	500

cDNA Synthesis Module

Component	100-rxn kit	500-rxn kit
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
E. Coli 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

Component	100-rxn kit	500-rxn kit
Platinum® SYBR® Green qPCR SuperMix-UDG*	2 × 1.25 ml	12.5 ml
50-mM Magnesium Chloride ($MgCl_2$)	1 ml	2 × 1 ml
20X Bovine Serum Albumin (BSA), UltraPure (1 mg/ml)	300 µl	1.25 ml
ROX Reference Dye	100 µl	500 µl

*SYBR® Green I, 60 U/ml Platinum® *Taq* 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, 400-µM dUTP, 40 U/ml UDG, and stabilizers.

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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
- Ice
- Pipettes
- Disposable gloves

Introduction

System Overview

The SuperScript® III Platinum® CellsDirect Two-Step qRT-PCR Kit with SYBR® Green 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® SYBR® Green qPCR 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® SYBR® Green qPCR SuperMix-UDG ensures optimal qPCR performance using SYBR® Green I dye, with excellent sensitivity and a linear dose response over a wide range of target concentrations.

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® SYBR® Green qPCR SuperMix-UDG ensures optimal sensitivity and performance in qPCR using SYBR® Green I fluorescent dye, with built-in carryover contamination protection and a linear dose response over a wide range of target concentrations

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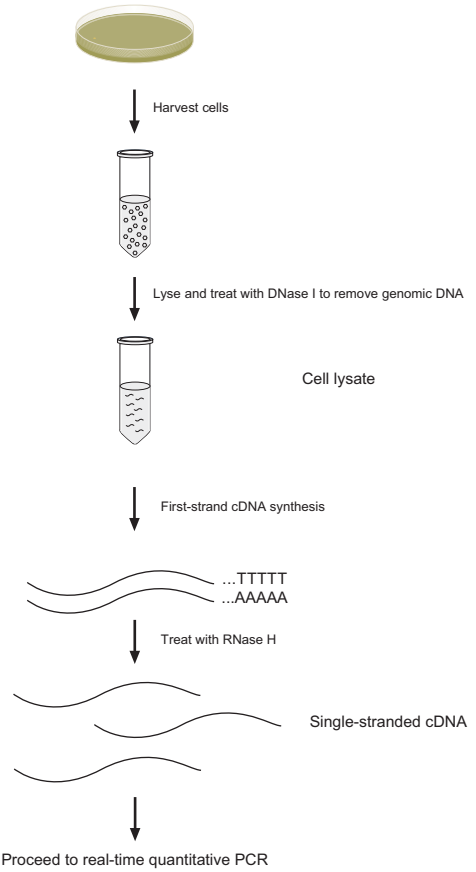
Diagram of cDNA Synthesis from Cell Lysate



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.

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Platinum® SYBR® Green qPCR SuperMix-UDG

Platinum® SYBR® Green qPCR 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 SYBR® Green I fluorescent dye, Platinum® *Taq* DNA polymerase, Mg^{++}, uracil-DNA glycosylase (UDG), proprietary stabilizers, and deoxyribonucleotide triphosphates (dNTPs), with dUTP instead of dTTP. The concentration of the SuperMix allows for the addition of primers and template.

SYBR® Green I is a fluorescent dye that binds directly to double-stranded DNA (dsDNA). In qPCR, as dsDNA accumulates, the dye generates a signal that is proportional to the DNA concentration and that can be detected using qPCR instruments. SYBR® Green I in this SuperMix formulation can quantify as few as 10 copies of a target gene in as little as 1 pg of template DNA or RNA. It has a broad dynamic range of six orders of magnitude, and is compatible with melting curve analysis.

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/µl)
- 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 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 temperate or in a 37°C incubator.
3. 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.
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 × *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 × *g* for 5 minutes to pellet.
9. Aspirate the PBS and resuspend the pellet in 500 µl 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/µl. 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 µl of Lysis Enhancer and 10 µl of Resuspension Buffer. **Note:** A master mix of Lysis Enhancer and Resuspension Buffer may be prepared for multiple reactions.
13. Transfer 1–2 µl of cells (<10,000 cells) to the PCR tube/well.

    Control Reaction: For the control reaction, add 1 µl 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.

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Note

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

- Seed cells in tissue culture wells so that 10 µl 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 µl of Lysis Enhancer for every 10 µl 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 µl of the buffer/enhancer mix to each well. For 24-well plates, add at least 110 µl 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 µl of the cell suspension to a 0.2-ml thin-walled PCR tube or plate well.

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

Continued on next page

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:

Component	Amount
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 cDNA yield.
4. Spin briefly, and add 4 µl 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:

Component	Amount
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
- Pipettes

The following materials are provided in the kit:

- Components of the qPCR module

Instrument Settings

Platinum® SYBR® Green qPCR SuperMix-UDG can be used 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®. Optimal cycling conditions will vary; refer to your instrument manual for operating instructions.

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

Primers

Primer selection is one of the most important parameters for qPCR when using a SYBR® Green detection system. To design primers, we strongly recommend using a primer design software program such as OligoPerfect™, available on the Web at www.invitrogen.com/oligos. In OligoPerfect™ designer, enter your target sequence and select *PCR: Detection* from the Application pulldown menu. Using primer design software will ensure that primers are specific for the target sequence and free of internal secondary structure, and avoid complementation at 3′-ends within each primer and with each other.

When designing primers, keep in mind that the amplicon length should be approximately 80–250 bp to optimize the efficiency of qPCR. 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.

Amplicon Size

For best results, the amplicon should be limited to 80–250 bp in size.

Note

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

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Melting Curve Analysis

Melting curve analysis should always 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.

Magnesium Concentration

Platinum® SYBR® Green qPCR SuperMix-UDG includes magnesium chloride at a final concentration of 3 mM. Optimal performance for any given target may require adjusting this level of magnesium. If necessary, use the 50-mM magnesium chloride solution included in the kit to increase the magnesium concentration.

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. To prepare a master mix of ROX and Platinum® SYBR® Green qPCR SuperMix-UDG:

1. Add ROX Reference Dye to Platinum® SYBR® Green qPCR 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 ROX/SuperMix-UDG mixture per 50 µl of reaction volume.

Note: Use of ROX Reference Dye is not supported on the iCycler™, Rotor-Gene™, Opticon®, and LightCycler® platforms.. ROX Reference Dye is not required on the ABI PRISM® 7900.

Bovine Serum Albumin (BSA)

BSA (ultrapure, non-acetylated) is included as a separate tube in each kit for use in LightCycler® reactions.

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 13.

Note: This cycling program is recommended as a starting point and guideline. Optimal cycling temperatures and times may vary for different target sequences, primer sets, and instruments.

After programming the instrument and preparing the reaction mix, follow the protocol on the following page to perform the reaction.

Note

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.

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® SYBR® Green qPCR 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
Autoclaved, distilled water[2]	to 42–49 µl	to 2100–2550 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, 200-µM dUTP, 1 U UDG

[2]Volume 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.

qPCR — Roche LightCycler®

Introduction

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

After programming the instrument and preparing the reaction mix, follow the protocol on the following page to perform the reaction.

Cycling Program

Program the LightCycler® 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

Program choice: Melting curve
Analysis mode: Melting curves
95°C, 0 seconds (20°C/second transition)
55°C, 15 seconds (20°C/second transition)
92°C, 0 seconds (0.1°C/second transition — continuous acquisition)
40°C, 0 seconds

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.

Component	1 rxn	34 rxns
Platinum® SYBR® Green qPCR SuperMix-UDG*	10 µl	340 µl
BSA, UltraPure (1 mg/ml)	1 µl	34 µl
Forward primer, 10 µM	1 µl	34 µl
Reverse primer, 10 µM	1 µl	34 µl
Autoclaved, distilled water	to 18 µl	to 612 µl

*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, 200-µM dUTP, 1 U UDG

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qPCR — Roche LightCycler®, continued

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 × *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.

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.
	Primer design is suboptimal	Verify your primer selection. We recommend using validated pre-designed primers or designing primers using dedicated software programs or primer databases.
	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	See your instrument manual for tips and troubleshooting.
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 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. We recommend using validated pre-designed primer sets or designing primers 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 gel 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.

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