Invitrogen™
life technologies

Instruction Manual

SuperScript™ III CellsDirect cDNA Synthesis System

Catalog Nos. 18080-200 and 18080-300

Version B
18 April 2005
25-0731

Table of Contents

Kit Contents and Storage

Shipping and Storage

Kit components are shipped on dry ice and should be stored at –20°C.

Kit Components

Catalog no. 18080-200 provides reagents for 25 reactions.
Catalog no. 18080-300 provides reagents for 100 reactions.

Component	25 Rxns	100 Rxns
Resuspension Buffer	250 µl	1 ml
RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/µl)	50 µl	200 µl
DNase I (1 U/µl)	125 µl	500 µl
10X DNase I Buffer	40 µl	160 µl
25 mM EDTA	30 µl	120 µl
Oligo(dT)$_{20}$ (50 µM)	50 µl	120 µl
10 mM dNTP Mix	25 µl	100 µl
SuperScript™ III RT (200 units/µl)	25 µl	100 µl
5X RT Buffer*	150 µl	600 µl
0.1 M DTT	50 µl	100 µl
E. Coli RNase H (2 U/µl)	30 µl	100 µl
HeLa Total RNA (10 ng/µl)	10 µl	10 µl
Forward Control Primer (10 µM)	10 µl	10 µl
Reverse Control Primer (10 µM)	10 µl	10 µl

* 5X RT Buffer composition: 250 mM Tris-HCl (pH 8.3, room temp.), 375 mM KCl; 15 mM $MgCl_2$

Note

DNA polymerase is not included in this kit. Recommended DNA polymerases and optional amplification protocols are provided starting on page 9.

Quality Control

This kit was verified in an end-point RT-PCR reaction using a serial dilution of HeLa cells and GAPDH primers, yielding a 1.18 kb PCR product. No template controls and negative reverse transcriptase controls were also prepared. Results were confirmed by gel electrophoresis.

Additional Products

Additional Products

The following related products are available from Invitrogen.

Product	Size	Cat. No.
SuperScript™ III Platinum® CellsDirect Two-Step qRT-PCR Kit	25 RT/100 qPCR 100 RT/500 qPCR	11737-030 11737-038
SuperScript™ III Platinum® CellsDirect Two-Step qRT-PCR Kit with SYBR® Green	25 RT/100 qPCR 100 RT/500 qPCR	11738-060 11738-068
CellsDirect Resuspension Buffer and Lysis Enhancer	10 ml/1 ml	11739-010
Platinum® *Taq* DNA Polymerase	100 reactions 250 reactions 500 reactions	10966-018 10966-026 10966-034
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions 500 reactions	11304-011 11304-029
Platinum® PCR SuperMix	100 reactions	11306-016
Platinum® PCR SuperMix High Fidelity	100 reactions	12532-016
E-Gel® Pre-cast Agarose Gels 0.8% Starter Pak 1.2% Starter Pak 2% Starter Pak 4% Starter Pak	 9 gels and base 9 gels and base 9 gels and base 9 gels and base	 G5000-08 G5000-01 G5000-02 G5000-04
100-bp DNA Ladder	50 µg	15628-019
1-Kb Plus DNA Ladder	250 µg 1,000 µg	10787-018 10787-026

Additional Materials Required

The following materials are provided by the user:

- Lysis Enhancer (optional; see page 4) (Catalog no. 11739-010)
- 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 Ca^{++} or Mg^{++}
- 0.2-ml thin-walled PCR tubes or 96-well PCR plates
- Ice
- Pipettes
- DNA polymerase and associated PCR reagents (see pages 9–11)
- Thermal cycler for PCR

Introduction

System Overview

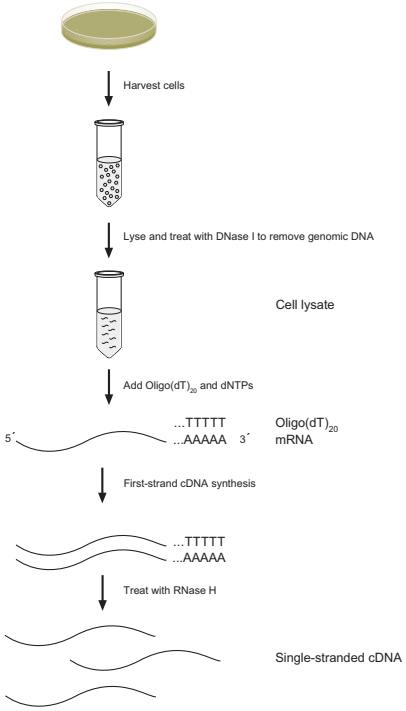
The SuperScript™ III CellsDirect cDNA Synthesis System is an optimized kit for synthesizing first-strand cDNA directly from mammalian cell lysate without first isolating the RNA. Lysis and reverse transcription are performed in the same tube, and the resulting first-strand cDNA is ready to use in cloning and PCR. For real-time quantitative RT-PCR, see the note on the following page.

In traditional RT-PCR, RNA is first isolated from cells in a time-consuming procedure that can lead to a loss of material. Using the SuperScript™ III 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.

This kit has been optimized for small cell samples, ranging from 10,000 cells down to a single cell (as measured by serial dilution). 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.

After synthesis, the first-strand cDNA can be amplified with specific primers by PCR without intermediate organic extractions or ethanol precipitations.

The diagram below outlines the procedure:



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Note

For real-time quantitative RT-PCR (qRT-PCR) from cell lysate, we recommend the SuperScript™ III Platinum® CellsDirect Two-Step qRT-PCR Kit (Catalog nos. 11737-030 and 11737-038) or the SuperScript™ III Platinum® CellsDirect Two-Step qRT-PCR Kit with SYBR® Green (Catalog nos. 11738-060 and 11738-068).

These kits include reagents and protocols that have been specifically optimized for real-time qRT-PCR.

Advantages of the Kit

This kit has the following advantages:

- Compatible with a wide range of mammalian cell types grown under different treatment conditions
- Single-tube format minimizes reagent loss, sample loss, and handling time
- Total lysate volume is used in first-strand 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
- Generates high-quality cDNA for use in a variety of applications, including cloning and PCR
- Simple protocol takes less than 2 hours

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.

Control RNA and Primers

The control RNA provided with this system consists of HeLa Total RNA (10 ng/µl). The Forward Control Primer and Reverse Control Primer provided with this kit are designed from the human GAPDH gene and produce a 1.18-kb PCR product.

Methods

Lysing Cells

Introduction

In this step, you lyse your cells in Resuspension Buffer or a Resuspension Buffer/Lysis Enhancer solution 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. The performance of this kit was verified using several different mammalian cell lines, including HeLa, COS-7, 293, Jurkat, CV1, and K562. Cells may be grown under 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:

- Lysis Enhancer (optional) (Catalog no. 11739-010)
- 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 Ca^{++} or Mg^{++}
- 0.2-ml thin-walled PCR tubes or 96-well PCR plates
- Ice
- Pipettes

The following materials are provided in the kit:

- Resuspension Buffer
- RNaseOUT™ (40 U/µl)
- DNase I, Amplification Grade (1 U/µl)
- 10X DNase I Buffer
- EDTA, 25 mM
- Optional: Control HeLa Total RNA

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Lysing Cells, continued

Lysis Enhancer

We recommend using Lysis Enhancer in the following procedures. Lysis Enhancer has been specially formulated for use with this kit to facilitate cell lysis. Catalog no. 11739-010 provides 1 ml of Lysis Enhancer and 10 ml of Resuspension Buffer (the additional Resuspension Buffer may be required for larger-volume tissue-culture wells).

For Cells in Tissue-Culture Wells: Prepare a 10:1 solution of Resuspension Buffer/Lysis Enhancer immediately before use (e.g., 10 µl of Resuspension Buffer to 1 µl of Lysis Enhancer).

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.

Control Reaction

For the control reaction, use 1 µl of the HeLa Total RNA provided in the kit instead of cell lysate.

Lysing Adherent Cells or Cells in Suspension

Use the following procedure for lysing adherent cell cultures in vessels larger than 24-well plate wells. 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.
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.

Procedure continued on next page

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Lysing Cells, continued

Lysing Adherent Cells or Cells in Suspension, continued

Procedure continued from previous page

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 10 µl of Resuspension Buffer. Then add 1 µl of Lysis Enhancer (recommended; see previous page) *or* 1 µl of RNaseOUT™ (40 U/µl).
13. Transfer 1–2 µl of cells (<10,000 cells) to the PCR tube/well.

    Control: For the control reaction, add 1 µl of the 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:** 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.

Amount of Resuspension Buffer in Tissue-Culture Wells

The following **minimum** volumes of Resuspension Buffer or Resuspension Buffer/Lysis Enhancer solution are required for lysing cells in tissue-culture wells:

Number of Wells in Tissue-Culture Plate	Volume of Resuspension Buffer (or Buffer/ Lysis Enhancer) per Well
24	100 µl
48	50 µl
96	10 µl

Higher volumes may be required. Cells should be completely covered by solution.

Note

Extra Resuspension Buffer may be required for lysing cells in larger-volume tissue-culture wells. Extra Resuspension Buffer is provided in catalog no. 11739-010, which includes 10 ml of Resuspension Buffer and 1 ml of Lysis Enhancer.

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Lysing Cells in Tissue-Culture Wells

Note: Seed cells in tissue-culture wells so that 10 µl of resuspended cells will yield the desired concentration.

For adherent cells grown in tissue-culture wells, 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 Resuspension Buffer or Resuspension Buffer/Lysis Enhancer solution (recommended; see page 4) to each well. See the table on page 5 for amounts. The buffer 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. If the cells are difficult to detach, incubate the plates at room temperature or 37°C for an additional 5 minutes, checking the cells under a microscope periodically.
5. Count the cells or estimate their density based on the seeding density (10 µl should contain <10,000 cells).
6. Transfer 10 µl of the cell suspension to a 0.2-ml thin-walled PCR tube or plate well.

   Control: For the control reaction, add 10 µl of Resuspension Buffer to a PCR tube or plate well, and then add 1 µl of Control HeLa Total RNA.
7. Add 1 µl of RNaseOUT™ (40 U/µl) to the PCR tube/well.
8. Transfer the tube/plate to an incubator or thermal cycler preheated to 75°C and incubate for 10 minutes. **Control:** For the control reaction, incubate for 3 minutes.
9. After incubation, spin briefly to collect the condensation, and proceed to **DNase I Digestion,** page 7.

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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 9, 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, and spin briefly to collect the contents.
3. Incubate for 5 minutes at room temperature. **Note:** A longer incubation time (up to 10 minutes) may be used for larger samples (>1,000 cells). However, incubation times exceeding 10 minutes can greatly reduce cDNA yield.
4. Spin briefly, and add 1.2 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 5 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 70°C
- Ice
- Pipettes

The following materials are provided in the kit:

- Oligo(dT)$_{20}$ (50 µM)
- 10 mM dNTP Mix
- 5X RT Buffer
- RNaseOUT™ (40 U/µl)
- SuperScript™ III RT (200 U/µl)
- 0.1 M DTT
- RNase H (2 U/µl)

First-Strand cDNA Synthesis

1. Place each tube from **DNase I Digestion,** Step 6, page 7, on ice, and add the following:

Component	Amount
Oligo(dT)$_{20}$ (50 mM)	2 µl
10 mM dNTP Mix	1 µl

2. Mix by gently pipetting up and down, and spin the tube briefly to collect the contents.
3. Incubate the tube at 70°C for 5 minutes. Spin the tube briefly to collect the condensation.
4. Place the tube on ice for 2 minutes, and then add the following:

Component	Amount
5X RT Buffer	6 µl
RNaseOUT™ (40 U/µl)	1 µl
SuperScript™ III RT (200 U/µl)*	1 µl
0.1 M DTT	1 µl

*For negative RT controls, use 1 µl of sterile, distilled water instead of SuperScript™ III RT

5. Mix by gently pipetting up and down, and spin the tube briefly to collect the contents.
6. Transfer the tube to a thermal cycler preheated to 50°C. Incubate for 50 minutes.
7. Inactivate the reaction at 85°C for 5 minutes.
8. Add 1 µl of RNase H (2 U/µl) to each tube and incubate at 37°C for 20 minutes. **Note:** This step is optional if you are amplifying short targets (<1.0 kb) in end-point PCR.
9. Chill the reaction on ice.
10. Store the single-stranded cDNA at –20°C, or proceed directly to PCR amplification.

PCR

Introduction

The first-strand cDNA generated using this kit can be used directly in PCR without additional purification. This section provides example protocols for PCR and high-fidelity PCR.

PCR Products

The following PCR products are available separately:

Product	Size	Cat. No.
Platinum® *Taq* DNA Polymerase	100 rxns	10966-018
	250 rxns	10966-026
	500 rxns	10966-034
	5000 rxns	10966-083
Platinum® *Taq* DNA Polymerase High Fidelity	100 rxns	11304-011
	500 rxns	11304-029
	5000 rxns	11304-102
Platinum® PCR SuperMix	100 rxns	11306-016
Platinum® PCR SuperMix High Fidelity	100 rxns	12532-016

PCR Enzymes

For amplifying the first-strand cDNA generated using this kit, we recommend Platinum® *Taq* DNA Polymerase for targets < 1.0 kb and Platinum® *Taq* DNA Polymerase High Fidelity for targets > 1.0 kb.

Platinum® *Taq* DNA Polymerase is recombinant *Taq* DNA polymerase complexed with proprietary Platinum® antibodies that block polymerase activity at ambient temperatures. Activity is restored after the denaturation step in PCR cycling at 94°C, providing an automatic "hot start" for *Taq* DNA polymerase in PCR. Hot starts in PCR provide increased sensitivity, specificity, and yield, while allowing assembly of reactions at room temperature. The use of Platinum® antibodies helps reduce PCR optimization requirements, reaction set-up and handling time, and contamination risk, thereby improving PCR results for templates up to 5 kb.

Platinum® *Taq* DNA Polymerase High Fidelity is a mixture of recombinant *Taq* DNA polymerase, *Pyrococcus* species *GB-D* polymerase, and Platinum® *Taq* antibody. Platinum® antibody complexes with *Taq* DNA polymerase and inhibits activity at ambient temperatures, allowing room-temperature setup. Activity is restored after the PCR denaturation step at 94°C, providing an automatic "hot start" for the enzyme and increasing specificity, sensitivity, and yield.

Pyrococcus species *GB-D* polymerase is a proofreading enzyme that possesses a 3′ to 5′ exonuclease activity. The enzyme mixture in Platinum® *Taq* DNA Polymerase High Fidelity results in a six-fold increase in fidelity over *Taq* DNA polymerase alone and allows amplification of simple and complex DNA templates over a large range of target sizes, up to 12 kb with no optimization.

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Note

- Since PCR is a powerful technique capable of amplifying trace amounts of DNA, take all appropriate precautions to avoid sample contamination.
- Annealing and extension conditions are dependent on primer T_m, and should be determined independently for each reaction.
- If PCR efficiency is not optimal, repeat the reaction with a primer titration from 100 to 500 nM (final conc.) in 100-nM increments.

PCR — Targets Up to 1 KB

The following protocol uses Platinum® *Taq* DNA Polymerase in a standard PCR reaction. Adjust the reaction size as needed. Optimal reaction conditions—including incubation times and temperatures, and concentrations of enzyme, primers, and $MgCl_2$—may vary.

Note: A concentration of 1.5 mM $MgCl_2$ is sufficient for most targets. For further optimization, prepare a titration from 1.5 mM to 3 mM in 0.25-mM increments.

1. Add the following components to a sterile 0.2- or 0.5-ml PCR tube or plate well at room temperature or on ice. For multiple reactions, prepare a master mix of common components.

Components	Volume	Final Conc.
10X PCR Buffer, Minus Mg	5 µl	1X
10 mM dNTP mixture	1 µl	0.2 mM each
50-mM $MgCl_2$	1.5 µl	1.5 mM
Sense primer (10 µM)	1 µl	0.2 µM
Antisense primer (10 µM)	1 µl	0.2 µM
cDNA from Step 10, page 8	2 µl	—
Platinum® *Taq* DNA Polymerase	0.4 µl	2.0 units*
Autoclaved, distilled water	to 50 µl	n/a

   *2.0 units are recommended for amplifying cDNA from the CellsDirect kit. In some cases, more enzyme may be required (up to 2.5 units).

2. Mix contents of the tubes and overlay with 50 µl of mineral or silicone oil, if necessary.
3. Cap the tubes and centrifuge briefly to collect the contents.
4. Incubate tubes in a thermal cycler at 94°C for 30 seconds to 2 minutes to denature the template and activate the enzyme.
5. Perform 30–40 cycles of PCR amplification as follows:

Denature	94°C for 15–30 seconds
Anneal	55–65°C for 30 seconds
Extend	72°C for 1 minute per kb

6. Maintain the reaction at 4°C after cycling. The samples can be stored at -20°C until use.
7. Analyze the products by agarose gel electrophoresis and visualize by ethidium bromide staining. Use appropriate molecular weight standards.

Continued on next page

PCR — Targets Above 1 KB

The following protocol uses Platinum® *Taq* DNA Polymerase High Fidelity. Adjust the reaction size as needed. Optimal reaction conditions—including incubation times and temperatures, and the concentrations of Platinum® *Taq* DNA Polymerase High Fidelity, primers, $MgSO_4$, and template DNA—may vary.

Note: A concentration of 2 mM $MgSO_4$ is sufficient for most targets. For further optimization, prepare a titration from 2 mM to 4 mM in 0.25-mM increments.

1. Add the following components to a sterile 0.2- or 0.5-ml PCR tube or plate well at room temperature or on ice. For multiple reactions, prepare a master mix of common components.

Component	Volume	Final Conc
10X High Fidelity PCR Buffer	5 µl	1X
10-mM dNTP mixture	1 µl	0.2 mM each
50-mM $MgSO_4$	2 µl	2 mM
Sense primer (10 µM)	1 µl	0.2 µM
Antisense primer (10 µM)	1 µl	0.2 µM
cDNA from Step 10, page 8	≥1 µl	—
Platinum® *Taq* High Fidelity	0.2 µl	1.0 unit*
Autoclaved, distilled water	to 50 µl	Not applicable

   *1.0 unit is sufficient for amplifying most targets. In some cases, more enzyme may be required (up to 2.5 units).

2. Mix contents of the tubes and overlay with 50 µl of mineral or silicone oil, if necessary.
3. Cap the tubes and centrifuge briefly to collect the contents.
4. Incubate tubes in a thermal cycler at 94°C for 30 seconds to 2 minutes to denature the template and activate the enzyme.
5. Perform 30–40 cycles of PCR amplification as follows:

Denature	94°C for 15–30 seconds
Anneal	55–65°C for 30 seconds
Extend	68°C for 1 minute per kb

6. Maintain the reaction at 4°C after cycling. The samples can be stored at -20°C until use.
7. Analyze the products by agarose gel electrophoresis and visualize by ethidium bromide staining. Use appropriate molecular weight standards.

Real-Time qRT-PCR

For real-time quantitative RT-PCR (qRT-PCR) from cell lysate, we recommend the SuperScript™ III Platinum® CellsDirect Two-Step qRT-PCR Kit (Catalog nos. 11737-030 and 11737-038) or the SuperScript™ III Platinum® CellsDirect Two-Step qRT-PCR Kit with SYBR® Green (Catalog nos. 11738-060 and 11738-068).

These kits include reagents and protocols that have been specifically optimized for real-time qRT-PCR.

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PCR — Control Reaction

The following protocol uses Platinum® *Taq* DNA Polymerase and the Control Primers provided in the kit.

1. Prepare a PCR mixture for each control reaction from Step 10, page 8. For each control reaction, add the following to a sterile 0.2- or 0.5-ml PCR tube or plate well at either room temperature or on ice:

Component	Volume
DEPC-treated water	38.1 µl
10X PCR buffer minus Mg^{++}	5 µl
50 mM $MgCl_2$	1.5 µl
10 mM dNTP mix	1 µl
Forward Control Primer (10 µM)	1 µl
Reverse Control Primer (10 µM)	1 µl
cDNA from control RNA/ negative RT control, Step 10, page 8	2 µl
Platinum® *Taq* DNA polymerase (5 units/µl)	0.4 µl
final volume	50 µl

2. Mix the contents of the tube. Centrifuge briefly to collect the reaction components.
3. Place reaction mixture in preheated (94°C) thermal cycler. Perform an initial denaturation step: 94°C for 2 min.
4. Perform 40 cycles of PCR:

Denature	94°C for 15 sec
Anneal	60°C for 30 sec
Extend	72°C for 1 min

   Note: For slow-ramping thermal cyclers, follow manufacturer's directions.
5. Upon completion, maintain reactions at 4°C.
6. Analyze 10 µl of each sample using agarose gel electrophoresis and ethidium bromide staining. A 1.18-kb band corresponding to at least 25 ng of product should be visible for the control sample. No band should be visible for the negative RT control sample.

Troubleshooting

Problem	Possible Cause	Suggested Solution
No bands after electrophoretic analysis of amplified products	Procedural error	Confirm that all steps were followed. Use the Control RNA to verify the efficiency of the first-strand reaction (see page 12 for 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. Confirm that RNaseOUT™ was added at the appropriate steps in the protocol. 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.
	Target mRNA contains strong transcriptional pauses	Use random hexamers (Cat. no. 48190-011) instead of oligo$(dT)_{20}$ in the first-strand reaction. Maintain an elevated temperature after the annealing step. Increase the temperature of first-strand reaction (up to 55°C). Use PCR primers closer to the 3´ terminus of the target cDNA.
	Too much first-strand product was used in PCR	Use no more than 5 µl of the first-strand product in PCR.
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 primers	Vary the annealing conditions. Use Platinum® *Taq* DNA Polymerase for automatic hot-start PCR. Optimize magnesium concentration for each template and primer combination.

Purchaser Notification

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References

Berger, S.L. and Kimmel, A.R. (1987) *Methods Enzymol* 152, 316.

Bracete, A.M., Mertz, L.M., Fox, D.K. (1999) *Focus*® 21, 38.

Chomczynski, P. (1993) *Biotechniques* Vol. 15, 532.

Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156.

Compton, T. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M., Gelfand, D., Sninsky, J., and White, T., eds.), p. 39, Academic Press, Inc.

D'Alessio, J. M., Gruber, C. E., Cain, C., and Noon, M. C. (1990) *Focus*® 12, 47.

Frohman, M.A., Dush, M.K, and Martin, G.R. (1988) *Proc. Nat. Acad. Sci USA* 85, 8998.

Gerard, G.F. (1994) *Focus*® 16, 102.

Gerard, G.F., D'Alessio, J.M., and Kotewicz, M.L. (1989) *Focus*® 11, 66.

Gerard, G.F., Schmidt, B.J., Kotewicz, M.L., and Campbell, J.H. (1992) *Focus*® 14, 91.

Hu, A.W., D'Alessio, J.M., Gerard, G.F., and Kullman, J. (1991) *Focus*® 13, 26.

Lee, C.C. and Caskey, T. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M., Gelfand, D., Sninsky, J., and White, T., eds.), p. 46, Academic Press, Inc.

Sambrook J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993) *Focus*® 15, 99.

Westfall, B., Sitaraman, K., Solus, J., Hughes, J., and Rashtchian, A. (1997) *Focus*® 19, 46.

Westfall, B., Sitaraman, K., Berninger, M., and Mertz, L.M. (1995) *Focus*® 17, 62.

Westfall, B., Sitaraman, K., Lee, J., Borman, J. and Rashtchian, A. (1999) *Focus*® 21, 49.

Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., Oka, M., and Imanaka, T. (1997) *Appl. Environ. Microbiol.* 63, 4504.

Sitaraman, K., Darfler, M., and Westfall, B. (1999) *Focus*® 21, 10.

Nathan, M., Mertz, L., Fox, D. (1995) *Focus*® 17, 78.

Schwabe, W., Lee, J.E., Nathan, M., Xu, R.H., Sitaraman, K., Smith, M., Potter, R.J., Rosenthal, K., Rashtchian, A., Gerard, G.F. (1998) *Focus*® 20, 30.

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