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QIAGEN® LongRange 2Step RT-PCR Handbook

For reliable and accurate long-range two-step RT-PCR up to 12.5 kb

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QIAGEN Sample and Assay Technologies

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Kit Contents

QIAGEN LongRange 2Step RT-PCR Kit	(10x RT; 20x PCR)	(50x RT; 100x PCR)
Catalog no.	**205920**	**205922**
No. of 20 µl RT* reactions and 50 µl long-range PCRs	**10x RT; 20x PCR**	**50x RT; 100x PCR**
LongRange RT Buffer, 5x	40 µl	200 µl
LongRange Reverse Transcriptase (1 reaction/µl)	10 µl	50 µl
Oligo-dT, 20 µM	60 µl	60 µl
LongRange PCR Enzyme Mix	8 µl	40 µl
LongRange RNase Inhibitor	5 µl	25 µl
LongRange PCR Buffer, 10x	100 µl	500 µl
Q-Solution®, 5x	400 µl	2 ml
dNTP Mix, 10 mM each	1x 20 µl, 1x 50 µl	2x 200 µl
$MgCl_2$	1.2 ml	1.2 ml
RNase-Free Water	1.9 ml	4x 1.9 ml
Handbook	1	1

* Reverse transcription.

Shipping and Storage

The QIAGEN LongRange 2Step RT-PCR Kit is shipped on dry ice. The kit, including all reagents and buffers, should be stored immediately upon receipt at –20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the products can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each component of the QIAGEN LongRange 2Step RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

The QIAGEN LongRange 2Step RT-PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the QIAGEN LongRange 2Step RT-PCR Kit, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Product Specifications

LongRange Reverse Transcriptase

The LongRange Reverse Transcriptase features a recombinant homodimeric viral reverse transcriptase for highly sensitive synthesis of full-length cDNAs up to 12.5 kb. Its intrinsic RNase H activity is post-translationally suppressed to a level that has no impact on full-length cDNA synthesis. This is triggered by a novel RNase H activity quencher, contained in the LongRange RT Buffer, which weakly binds to the cDNA:RNA hybrid–binding site of the reverse transcriptase preventing degradation of the RNA during synthesis. This results in reduced RNase H activity and synthesis of full-length cDNA.

LongRange PCR Enzyme Mix

LongRange PCR Enzyme Mix is a mixture of highly pure recombinant thermostable DNA polymerases and processivity-enhancing factors, cloned in *E. coli*.

Concentration:	5 units/µl; refers to the amount of *Taq* DNA polymerase per microliter of LongRange Enzyme Mix, although the specific activity (by nucleotide incorporation) of *Taq* DNA polymerase in combination with the other enzymes is higher than the specific activity of pure *Taq* DNA polymerase of the same concentration.
5'→3' exonuclease activity:	Yes
3'→5' exonuclease activity:	Yes
Extra A addition (terminal transferase activity):	Yes

Buffers and reagents

LongRange RT Buffer, 5x	5x concentrated, contains 25 mM Mg^{2+}
LongRange PCR Buffer, 10x:	10x concentrated, contains 25 mM Mg^{2+}
Q-Solution:	5x concentrated
dNTP Mix:	10 mM of each: dATP, dCTP, dGTP, dTTP
RNase-free water:	Ultrapure quality, PCR-grade
Enzyme storage buffer:	LongRange Enzyme Mix: Tris·Cl pH 8.0 (at 25°C), KCl, EDTA, DTT, 50% glycerol, Tween® 20, Igepal® CA-630

Introduction

The QIAGEN LongRange 2Step RT-PCR Kit includes all reagents needed for high fidelity long-range two-step RT-PCR. The unique enzyme formulations, innovative buffer solutions, and streamlined protocols provide easy handling and optimized results. The kit combines the features and benefits of the QIAGEN LongRange PCR Kit with an efficient reverse transcription step, in which a recombinant homodimeric viral reverse transcriptase synthesizes full-length cDNAs up to 12.5 kb.

LongRange RT Buffer

LongRange RT Buffer is specially formulated to provide reverse transcription at elevated temperatures, resulting in improved denaturation of difficult templates with only minimal optimization. The novel RNase H quencher binds weakly to the double-stranded binding site of the reverse transcriptase, preventing selective degradation of the RNA during synthesis and leading to synthesis of full-length cDNA (see Figure 1).

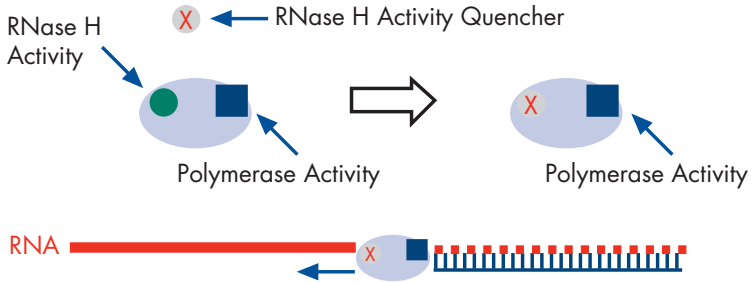


Figure 1. Full length cDNA amplification due to unique RNase H activity quencher. The RNase H Activity Quencher (a component of the RT Buffer) binds to the cDNA/RNA hybrid–binding site of the reverse transcriptase, preventing degradation of the RNA during synthesis. This results in reduced RNase H activity and the amplification of full-length cDNA up to 12.5 kb.

LongRange PCR enzymes

The LongRange PCR Enzyme Mix is a blend of thermostable DNA polymerases with proofreading-assisted fidelity and a high extension rate. In combination with LongRange PCR Buffer, the LongRange PCR Enzyme Mix guarantees robust amplification of very long cDNAs up to 12.5 kb in length. Amplification products generated using the QIAGEN Long-Range 2Step RT-PCR Kit can be directly cloned into TA/UA cloning vectors with high efficiency.

Q-Solution

The QIAGEN LongRange PCR Kit includes Q-Solution, an innovative PCR additive that facilitates amplification of difficult templates by modifying the melting behavior of DNA. This unique reagent often enables or improves a suboptimal PCR caused by templates that have a high degree of secondary structure or that are GC-rich. Unlike other commonly used PCR additives such as DMSO, Q-Solution is used at just one working concentration, is nontoxic, and PCR purity is guaranteed. Adding Q-Solution to the PCR does not compromise PCR fidelity. For further information, refer to the protocol for long-range PCR using Q-Solution, page 20.

Applications

The QIAGEN LongRange 2Step RT-PCR Kit is suitable for long-range two-step RT-PCR applications such as cloning and gene expression analysis. The high sensitivity and accuracy make this kit highly suitable for amplifying a wide range of targets, even those of very low abundance.

Applications of the QIAGEN LongRange 2Step RT-PCR System include amplification of:

- Long cDNA templates from RT reactions (all targets in the range 0.1–10 kb)
- Very long targets (cDNA >10 kb)
- GC-rich and other difficult templates

Starting template

Reverse transcriptases are used in vitro for first-strand synthesis of cDNA with an RNA template. The efficiency of the reaction is highly dependent on the quality and quantity of the RNA template. Intact RNA is essential for good results: even trace amounts of contaminating RNases in the sample can cause RNA cleavage, resulting in shortened cDNA products. Chemical impurities such as proteins, poly-anions (e.g., heparin), salts, EDTA, ethanol, phenol, and other solvents can affect the activity and processivity of the reverse transcriptase. To ensure reproducible and efficient reverse transcription, it is important to determine the quality and quantity of the starting RNA (see Appendix A, page 33).

For best results, we recommend starting with RNA purified using silica-gel–membrane technology. For example, RNeasy® Kits, the PAXgene™ Blood RNA Kit, QIAamp® Viral RNA Mini Kits, and the QIAamp RNA Blood Mini Kit can be used to isolate RNA from a variety of starting materials and provide high-quality RNA that is well suited for reverse transcription and RT-PCR. Alternatively, high-quality mRNA can be used, for example, purified using Oligotex® mRNA and Oligotex Direct mRNA Kits (see page 47 for ordering information).

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For all protocols

- Ice
- Pipets and pipet tips (use of pipet tips with hydrophobic filters is strongly recommended; see Appendix G, page 41)
- Thermal cycler
- PCR tubes (use thin-walled 0.2 ml PCR tubes recommended by the manufacturer of your thermal cycler)
- Primers and template RNA or cDNA
- For thermal cyclers without a heated lid: mineral oil
- For analysis of PCR products: agarose-gel electrophoresis system

For reverse transcription using LongRange Reverse Transcriptase

- Microcentrifuge
- Vortexer

Important Notes

Guidelines for two-step RT-PCR

In two-step RT-PCR, reverse transcription and PCR are performed sequentially in two separate reaction tubes. Use of oligo-dT primers is strongly recommended for the RT step in long-range two-step RT-PCR. First carry out a reverse-transcription reaction to generate cDNA (up to 12.5 kb in length), following the protocol on page 13 and using 50 ng to 4 µg of total RNA. Use an aliquot of the finished reverse-transcription reaction for PCR. To avoid PCR inhibition, the aliquot should contribute no more than 1/10 of the final PCR volume. For example, for a 50 µl PCR assay, use ≤5 µl of the finished reverse-transcription reaction.

Selection of PCR protocol

The LongRange 2Step RT-PCR Kit can be used to amplify cDNA of various sizes. The most suitable protocol for different templates is shown in Table 1.

Table 1. PCR Protocol Selection According to Template Size

Size and nature of template	Protocol for the PCR step
cDNA targets up to 10 kb	Protocol for long-range PCR, page 17
cDNA targets up to 10 kb with GC-rich regions or other difficult templates	Protocol for long-range PCR using Q-Solution, page 20
cDNA targets >10 kb	Protocol for very long-range PCR, page 23

Template RNA

- Ensure that the template RNA is of sufficiently high quality and is not degraded (for further details, see Appendix A, page 33).

Primers

- Primers should have annealing temperatures above 60°C, as determined by the 4+2 rule (see Appendix B, page 36 for further information).

Amplification

- Use thin-walled 0.2 ml PCR tubes recommended by the manufacturer of your thermal cycler, to ensure that the tubes fit correctly in the PCR block.

Agarose gel analysis of PCR products

- Analyze RT-PCR products on agarose gels using appropriate running conditions (see Appendix E, Table 16, page 40 for further information).

Protocol: Reverse Transcription Using LongRange Reverse Transcriptase

This protocol constitutes the first step of two-step RT-PCR

Important points before starting

- The protocol is optimized for use with 50 ng – 4 µg of total RNA or 1–100 ng of mRNA.
- Set up all reactions on ice to avoid premature synthesis of cDNA and to minimize the risk of RNA degradation.

RNA integrity, storage, and stability

The integrity and quality of template RNA is critical for successful RT-PCR analysis and cDNA synthesis.

To minimize RNA hydrolysis during storage:

- Store working solutions of RNA in small aliquots at –80°C
- For long-term stability, store the RNA as a precipitate under 96% ethanol at –80°C

To check the integrity of RNA:

- Run a denaturing agarose gel (Sambrook, J. and Russell, D.W. [2001] *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press) or use the QIAxcel system (cat. no. 9001421).
- On a denaturing gel, high-molecular-weight mRNA should be visible as a background smear of up to 8–9 kb
- The 28S rRNA band of intact RNA should be twice as intense as the 18S rRNA band

For further recommendations on storage, quantification, and determination of quality of RNA, see Appendix A, page 33.

Genomic DNA contamination:

Contamination of RNA by even trace amounts of genomic DNA can lead to poor results in reverse transcription. To minimize the effect of genomic DNA:

- Include a "minus reverse transcriptase" control reaction in your experiment
- Treat the RNA preparation with RNase-free DNase*

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Wherever possible, design primer pairs spanning an intron sequence or annealing at the junction of two consecutive exons

For the subsequent PCR, be sure to:

- Set up all reaction mixtures in an area separate from those used for DNA preparation or for analysis of RT-PCR products
- Use reagents and pipets dedicated to setup of reverse transcription and PCR
- Use disposable pipet tips containing hydrophobic filters to minimize the risk of cross contamination

Things to do before starting

- Separate denaturation and annealing steps for cDNA synthesis are usually not necessary. However, a denaturation step may be advisable for some RNAs with a high degree of secondary structure. If so, denature the RNA in RNase-free water before reaction setup by incubating the RNA for 5 min at 65°C, then placing immediately on ice. Do not denature the RNA in the reaction mix.

Procedure

1. **Thaw template RNA on ice. Thaw oligo-dT, 5x LongRange RT Buffer, dNTP Mix, and RNase-free water at room temperature (15–25°C), and store on ice immediately after thawing.**

   Important: Prepare the reaction mix and set up the reactions on ice.

   After thawing, mix each solution by vortexing and centrifuge briefly to collect residual liquid from the sides of the tubes.

2. **Prepare a master mix on ice according to Table 2.**

Table 2. Composition of Reverse-Transcription Master Mix

Component	Volume in each reaction	Final concentration
Reaction mix		
LongRange RT Buffer, 5x	4 µl	1x
dNTP mix, (10 mM each)	2 µl	1 mM of each dNTP
Oligo-dT (20 µM)*	1 µl	1 µM
LongRange RNase inhibitor (4 U/µl)	0.2 µl	0.04 U/µl
LongRange Reverse Transcriptase	1 µl	1x
RNase-free water	Variable	
Template RNA		
Added at step 5	Variable	50 ng – 4 µg per 20 µl reaction
Total volume	**20 µl**	

* If using a gene-specific primer, a final concentration of 1 µM is recommended. Use of random oligomer primers is not recommended for long-range RT-PCR.

3. **Mix the master mix thoroughly and carefully by vortexing for no more than 5 s, and store on ice. Centrifuge the master mix briefly to collect residual liquid from the walls of the tube.**

   The master mix contains all components required for first-strand synthesis except the template RNA. If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reverse-transcription reactions to be performed.

The protocol is optimized for use with 50 ng to 4 µg of total RNA. For >4 µg RNA, scale up the reaction linearly according to the total amount of RNA present to the appropriate volume. For example, for reverse transcription of 8 µg RNA, double the volumes of all reaction components to give a final reaction volume of 40 µl.

4. **If setting up more than one reverse-transcription reaction, dispense appropriate volumes of master mix into individual reaction tubes and store the tubes on ice.**
5. **Add template RNA to each tube containing master mix and mix thoroughly and carefully by vortexing for no more than 5 s.**

   Centrifuge the tubes briefly to collect residual liquid from the walls of the tubes.
6. **Incubate for 90 min at 42°C.**

   For synthesis of full-length cDNA up to 12.5 kb, the temperature of the reverse transcription should not exceed 42°C. The LongRange RT buffer composition provides efficient resolution of all secondary structures in RNA at this temperature.
7. **Inactivate the enzyme by heating at 85°C for 5 min.**
8. **If proceeding immediately with PCR, store the reverse-transcription reactions on ice and add an aliquot of the completed reaction to the PCR mix, following the appropriate protocol on page 17, 20, or 23.**

   If more PCR amplifications are required than are supplied with the kit, the QIAGEN LongRange PCR Kit can be purchased separately (see Ordering Information on page 44).

   Reverse-transcription reactions can be stored long-term at –20°C.

Protocol: Long-Range 2Step RT-PCR

This protocol is constitutes the second step ot two-step RT-PCR and allows amplification of cDNA targets up to approximately 10 kb.

Important points before starting

- Read the Important Notes on page 11 before starting the protocol.
- Set up all reactions on ice.
- Always use an elongation temperature of 68°C.
- Always use denaturation conditions of 93°C for 15 s. Do not exceed this temperature.
- Use a final dNTP concentration of 500 µM.

Procedure

1. **Thaw 10x LongRange PCR Buffer, dNTP mix, primer solutions, and RNase-free water.**

   Mix the solutions thoroughly before use.

2. **Prepare a reaction mix as shown in Table 3.**

   We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR Buffer.

   Important: Set up all reactions on ice.

Table 3. Composition of Reaction Mix for Long-Range PCR (0.1–10 kb)

Component	Volume in each reaction	Final concentration
Reaction mix		
LongRange PCR Buffer with Mg^{2+}, 10x	5 µl	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5 µl	500 µM of each dNTP
Primer A	Variable	0.4 µM
Primer B	Variable	0.4 µM
RNase-free water	Variable	
LongRange PCR Enzyme Mix	0.4 µl	2 units per 50 µl reaction
Template cDNA		
Added at step 4	Variable	50–500 ng
Total volume	**50 µl**	

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**
4. **Add 50–500 ng of cDNA template to each tube containing reaction mix.**

   The volume of cDNA added as template from the RT reaction should not exceed 10% of the final PCR volume. See Appendix C, page 39, for more information about RT-PCR.
5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 4.**

   When using a thermal cycler with a heated lid, do not use mineral oil.

Table 4. Cycling Protocol for Long-Range PCR (0.1–10 kb)

			Comments
Initial activation step:	3 min	93°C	Initial denaturation of template cDNA.
3-step cycling:			
Denaturation	15 s	93°C	Do not exceed this temperature.
Annealing	30 s	62°C	Approximately 5°C below T_m of primers (see Appendix B, Table 12, page 36).
Extension	1 min/kb	68°C	Use an extension time of 1 min per kilobase of cDNA.
Number of cycles	35		Amplification for 35 cycles will produce satisfactory results in most cases. However, the optimum number of cycles depends on the amount of template DNA. See Appendix C, page 39 for guidelines.
End of PCR cycling:	Indefinite	4°C	

6. **For a simplified hot start, place the tubes immediately into a thermal cycler that is preheated to 93°C and start the cycling program as outlined in Table 4.**

   Use the simplified hot start to ensure PCR specificity.

   After amplification, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

7. **Analyze the samples using an appropriate detection system such as agarose gel electrophoresis.**

   See Appendix E, Table 16, page 40, for choosing the optimal percentage of agarose.

   A TA/UA cloning system can be used for direct cloning of amplified fragments (see Appendix H, page 42).

Protocol: Long-Range 2Step RT-PCR Using Q-Solution

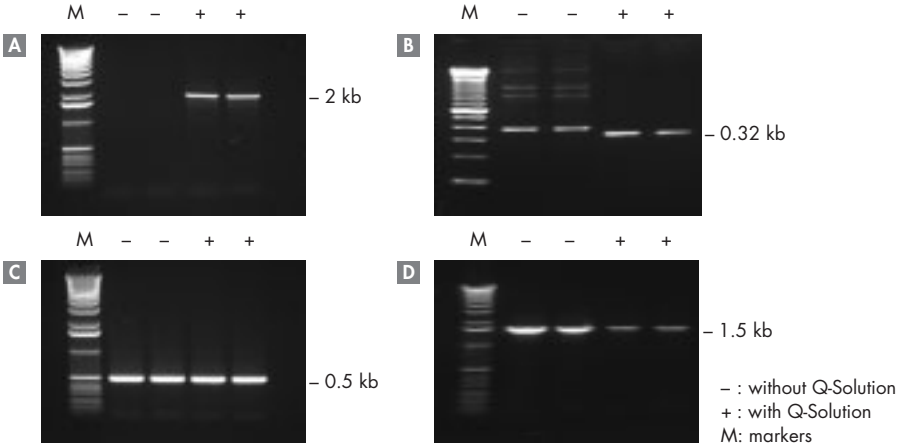
This protocol constitutes the second step of two-step RT-PCR and utilizes Q-Solution with cDNA targets up to approximately 10 kb in size. Q-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. When using Q-Solution, the following effects may be observed, depending on the individual PCR assay:

Case A: Q-Solution enables amplification of a reaction that previously failed.

Case B: Q-Solution increases PCR specificity in certain primer–template systems.

Case C: Q-Solution has no effect on PCR performance.

Case D: Q-Solution causes reduced efficiency or failure of a previously successful amplification reaction. In this case, addition of Q-Solution disturbs the previously optimal primer–template annealing. Therefore, when using Q-Solution for the first time for a particular primer–template system, always perform reactions in parallel with and without Q-Solution.



Important points before starting

- Read the Important Notes on page 11 before starting the protocol.
- Set up all reactions on ice.
- Always use an elongation temperature of 68°C.
- Always use denaturation conditions of 93°C for 15 s. Do not exceed this temperature.
- Use a final dNTP concentration of 500 µM.
- When using Q-Solution for the first time with a particular primer–template pair, always perform parallel reactions with and without Q-Solution; this recommendation should also be followed if another PCR additive (such as DMSO) was previously used for a particular primer-template pair.

Procedure

1. **Thaw 10x LongRange PCR Buffer, dNTP mix, primer solutions, and Q-Solution.**

   Mix the solutions thoroughly before use.

2. **Prepare a reaction mix according to Table 5.**

   We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR Buffer.

   Important: Set up all the reactions on ice.

Table 5. Composition of Reaction Mix for Long-Range PCR Using Q-Solution (0.1–10 kb)

Component	Volume in each reaction	Final concentration
Reaction mix		
LongRange PCR Buffer with Mg^{2+}, 10x	5 µl	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5 µl	500 µM of each dNTP
5x Q-Solution	10 µl	1x
Primer A	Variable	0.4 µM
Primer B	Variable	0.4 µM
RNase-free water	Variable	
LongRange PCR Enzyme Mix	0.4 µl	2 units per 50 µl reaction
Template cDNA		
Added at step 4	Variable	50–500 ng
Total volume	50 µl	

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**
4. **Add 50–500 ng of template cDNA to each tube containing reaction mix.**

   The volume of cDNA added as template from the RT reaction should not exceed 10% of the final PCR volume. See Appendix C, page 39, for more information about RT-PCR.

5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 6.**

   When using a thermal cycler with a heated lid, do not use mineral oil.

Table 6. Cycling Protocol for Long-Range PCR Using Q-Solution (0.1–10 kb)

			Comments
Initial activation step:	3 min	93°C	Initial denaturation of template cDNA.
3-step cycling:			
Denaturation	15 s	93°C	Do not exceed this temperature.
Annealing	30 s	62°C	Approximately 5°C below T_m of primers (see Appendix B, Table 12, page 36).
Extension	1 min/kb	68°C	Use an extension time of 1 min per kilobase DNA for genomic DNA targets For targets of low complexity such as phage or plasmid DNA, use 45 s per kilobase DNA.
Number of cycles:	35		Amplification for 35 cycles will produce satisfactory results in most cases. However, the optimum number of cycles depends on the amount of template DNA. See Appendix C, page 39 for guidelines.
End of PCR cycling:	Indefinite	4°C	

6. **For a simplified hot start, place the tubes immediately into a thermal cycler that is preheated to 93°C and start the cycling program as outlined in Table 6.**

   Use the simplified hot start to ensure PCR specificity.

   After amplification, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

7. **Analyze the samples using an appropriate detection system such as agarose gel electrophoresis.**

   See Appendix E, Table 16, page 40, for choosing the optimal percentage of agarose.

   A TA/UA cloning system can be used for direct cloning of amplified fragments (see Appendix H, page 42).

Protocol: Very Long-Range PCR

This protocol constitutes the second step of two-step RT-PCR and allows amplification of cDNA targets longer than 10 kb.

Important points before starting

- Read the Important Notes on page 11 before starting the protocol.
- Set up all reactions on ice.
- Primers should have annealing temperatures above 60°C, as determined by the 4+2 rule (see Appendix B, page 36 for further information).
- Always use an elongation temperature of 68°C.
- Always use denaturation conditions of 93°C for 15 s. Do not exceed this temperature.
- Use a final dNTP concentration of 500 µM.
- For difficult targets, the use of 1x Q-Solution may improve results.

Procedure

1. **Thaw 10x LongRange PCR Buffer, dNTP mix, primer solutions, and optionally Q-Solution.**

   Mix the solutions thoroughly before use.

2. **Prepare a reaction mix according to Table 7.**

   We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR Buffer.

   Important: Set up all reactions on ice.

Table 7. Composition of Reaction Mix for Very Long-Range PCR (>10 kb)

Component	Volume in each reaction	Final concentration
LongRange PCR Buffer with Mg^{2+}, 10x	5 µl	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5 µl	500 µM of each dNTP
Optional: 5x Q-Solution	10 µl	1x
Primer A	Variable	0.4 µM
Primer B	Variable	0.4 µM
RNase-free water	Variable	
LongRange PCR Enzyme Mix	0.4 µl	2 units per 50 µl reaction
Template cDNA, added at step 4	Variable	100–500 ng
Total volume:	**50 µl**	

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**
4. **Add template cDNA to each tube containing reaction mix, following the amounts recommended in Table 7.**

   The volume of cDNA added as template from the RT reaction should not exceed 10% of the final PCR volume.
5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 8.**

   When using a thermal cycler with a heated lid, do not use mineral oil.

Table 8. Cycling Protocol for Very Long-Range PCR (>10 kb)

			Comments
Initial activation step:	3 min	93°C	Initial denaturation of template cDNA.
3-step cycling: 38 cycles total*			
First 10 cycles:			
Denaturation	15 s	93°C	Do not exceed this temperature.
Annealing	30 s	62°C	Approximately 5°C below T_m of primers (see Appendix B, Table 12, page 36).
Extension	1 min/kb	68°C	Use an extension time of approximately 1 min per kilobase DNA.
Next 28 cycles:			
Denaturation	15 s	93°C	Do not exceed this temperature.
Annealing	30 s	62°C	Approximately 5°C below T_m of primers (see Appendix B, Table 12, page 36).
Extension	1 min/kb + 20 s in each additional cycle†	68°C	Use an extension time of approximately 1 min per kilobase DNA.

* Amplification for 38 cycles will produce satisfactory results in most cases. However, the optimum number of cycles depends on the amount of template DNA. See Appendix C, page 39 for guidelines.

† Program an extended extension time, referred to as "Time Increment", in which the extension time is increased by increments of 20 s in each cycle. For example, for a 10 kb fragment, program an extension time of 10 min 20 s in the 11th cycle, 10 min 40 s in the 12th, 11 min in the 13th, etc.

6. **For a simplified hot start, place the tubes immediately into a thermal cycler that is preheated to 93°C and start the cycling program as outlined in Table 8.**

   Use the simplified hot start to ensure PCR specificity.

   After amplification, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

7. **Analyze samples using an appropriate detection system such as agarose gel electrophoresis**

   See Appendix E, Table 16, page 40, for choosing the optimal percentage of agarose and running conditions.

   A TA/UA cloning system can be used for direct cloning of amplified fragments (see Appendix H, page 42).

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx . The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and suggestions
Little or no cDNA product in reverse-transcription reaction		
a)	Incorrect reaction setup	Ensure the reaction is set up on ice.
b)	Incorrect reaction temperature	Reverse transcription should be carried out at 42°C. Check the temperature of your heating block or water bath. In rare cases, when analyzing RNAs with a very high degree of secondary structure, it may be advantageous to increase the temperature up to 50°C.
c)	Pipetting error or missing reagent	Check the pipets used for experimental setup. Mix all reagents well after thawing, store on ice immediately after thawing, and repeat the reverse-transcription reaction.
d)	Poor quality or wrong amount of template	Check the concentration, integrity, and purity of starting template (see Appendix A, page 33). Mix well after thawing the RNA template, and use LongRange RNase inhibitor at a final concentration of 0.4 U/µl in the assay. Even minute amounts of RNases can affect the length of synthesized cDNAs and resulting sensitivity in RT-PCR, particularly with small amounts of RNA template.
e)	RNA concentration too high or too low	LongRange Reverse Transcriptase is designed for use with 50 ng – 4 µg RNA. With >4 µg RNA, scale up the reaction linearly to the appropriate volume.

		Comments and suggestions
f)	Incorrect nucleotide concentration or nucleotide degradation	Use the dNTP Mix provided with the kit. Using incorrect nucleotide concentrations can reduce the amount of cDNA product. Nucleotides will degrade if they are stored at room temperature (15–25°C).
g)	Incorrect primers	Oligo-dT primers rather than random primers in the reverse-transcription reaction are strongly recommended for amplification of long cDNA species.
h)	Incorrect denaturation conditions	Usually, denaturation of the RNA–primer mix is not necessary, but in some cases, denaturation of the RNA template allows more efficient priming. If so, denature the RNA in RNase-free water (provided with the kit). High denaturation temperatures (>65°C) or prolonged denaturation times (>5 min) can affect the integrity of RNA, resulting in shortened cDNA products.
i)	Incubation time too short	The standard reverse-transcription reaction requires a 90 min incubation. In rare cases, when analyzing RNAs with a very high degree of secondary structure, it may be advantageous to increase the incubation time to 2 h.
j)	Degraded RNA template	Check the integrity of the RNA template on a denaturing agarose gel.
k)	RNA of interest is not present in the sample	Verify that the RNA of interest is present.
l)	RNase contamination	Use only proven nuclease-free consumables (pipet tips, tubes, etc.). Decontaminate your work area from RNases before setting up the reverse-transcription reaction. Repeat the experiment using freshly isolated RNA and new consumables that have not been opened before.
Little or no PCR product		
a)	Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and template.

		Comments and suggestions
b)	Insufficient template	Increase the amount of template used in PCR, using the amounts recommended in the relevant protocols as a starting point. Consider the efficiency of the reverse transcriptase reaction, which is usually between 10 and 30%. See Appendix C, page 39, for information on template amounts and corresponding cycle numbers and enzyme concentrations. High quality templates are essential for amplification of long targets.
c)	PCR conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution, following the protocol on page 20.
d)	Primer concentration not optimal	Use a concentration of 0.4 µM of each primer. For calculation of primer concentration, see Appendix B, page 36.
e)	Enzyme concentration too low	Increase enzyme concentration up to a maximum of 2.5 U per 50 µl. If necessary, increase the amount of LongRange PCR Enzyme Mix in steps of 0.5 U. See Appendix C, Table 15, page 39 for further suggestions.
f)	Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles. Refer to the protocols and to Appendix C, page 39, to optimize the cycle numbers and enzyme concentrations for different amounts of template.
g)	Problems with template	Check the concentration, storage conditions, and quality of the template (see Appendix A, page 33). If necessary, make new serial dilutions of template nucleic acid from stock solutions and repeat the PCR using the new dilutions. Degraded template nucleic acid is not suitable for amplification of long targets.

		Comments and suggestions
h)	Incorrect annealing temperature or time	Use an annealing temperature 5°C below the T_m of your primers. See Appendix B, page 36, for how to determine the annealing temperature of your primers. Difficulties in determining the optimal annealing temperature can often be overcome by performing a gradient PCR (see Appendix D, page 39). Use the optimized annealing time of 30 s.
i)	Incorrect denaturation temperature or time	Denaturation should be at 93°C for 15 s. Ensure that the initial incubation for 3 min at 93°C was performed as described in step 5 of the PCR protocols (pages 18, 21, and 24)
j)	Primer design not optimal	Review primer design (see Appendix B, page 36).
k)	Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.
l)	Use of incorrect consumables	Check the fit of the PCR tube caps in the thermal cycler. Poor thermal contact prevents effective temperature transfer. Use only consumables recommended by the manufacturer of your thermal cycler.
m)	Air bubbles in PCR tube	Do not allow air bubbles to become trapped after mixing the reaction master mixes. Air bubbles prevent homogeneous temperature distribution throughout the reaction volume.
n)	Evaporation during thermal cycling	Check the fit of PCR tube caps or sealing foils on PCR plates. Long-range PCR is especially sensitive to evaporation.
Product is multibanded		
a)	PCR cycling conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution, following the protocol on page 20.

		Comments and suggestions
b)	Annealing temperature too low or annealing time incorrect	Use an annealing temperature 5°C below the T_m of your primers. See Appendix B, page 36, for how to determine the annealing temperature of your primers. Difficulties in determining the optimal annealing temperature can often be overcome by performing a gradient PCR (see Appendix D, page 39). First use the optimized annealing time of 30 s. If this is unsuccessful, reduce the annealing time in decrements of 10 s to a minimum of 10 s.
c)	Incorrect extension time	Adjust the length of the extension step according to the size of the expected PCR product (see Tables 4, 7, and 8).
d)	Primer concentration not optimal or primers degraded	Use a concentration of 0.4 µM of each primer. For calculation of primer concentration, refer to Appendix B, page 38. Check that primers have not degraded by analysis on a denaturing polyacrylamide gel,* particularly when performing highly sensitive PCR.
e)	Primer design not optimal	Review primer design (see Appendix B, page 36). Design new or longer primers.
f)	Q-Solution not used	Repeat the experiment, performing parallel reactions with and without Q-Solution (see Protocol: Long-Range PCR Using Q-Solution, page 20).
g)	Template concentration too high	Reduce the amount of template DNA.
h)	Too many cycles	Reduce the number of cycles in steps of 2.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

		Comments and suggestions
Product is smeared		
a)	Insufficient starting template	Increase the amount of template, using the amounts suggested in the relevant protocols as a starting point. Also see Appendix C, page 39, for information on template amounts and corresponding cycle numbers and enzyme concentrations.
b)	Poor analysis by agarose gel electrophoresis	Prepare agarose gels using a suitable concentration of agarose and run gels under appropriate conditions (see Table 16, page 40).
c)	Incorrect enzyme concentration	Reduce the amount of enzyme in decrements of 0.5 U.
d)	Mg^{2+} concentration not optimal	Use an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR buffer. In very rare cases, an increased Mg^{2+} concentration may improve PCR performance. Increase the concentration of Mg^{2+} ions in increments of 0.25 mM.
e)	Suboptimal ratio of Mg^{2+} ions:dNTP	Check the ratio of Mg^{2+} ions:dNTPs. A final dNTP concentration of 500 µM requires 2.5 mM Mg^{2+} ions, as recommended in the protocols.
f)	Primer design not optimal	Review primer design (see Appendix B, page 39).
g)	Too many cycles	Reduce the number of cycles in steps of 4.
h)	Elongation step too short	Increase the length of the elongation step. Increase the length of the elongation step. Use a minimum of 1min/kb. For very long targets see protocol on page 23.
Poor PCR fidelity		
a)	Insufficient starting template	Increase the amount of template used, using the amounts suggested in the relevant protocols as a starting point. Also see Appendix D, page 39, for information on template amounts and corresponding cycle numbers and enzyme concentrations.

		Comments and suggestions
b)	Mg^{2+} concentration too high	Optimal PCR fidelity using LongRange PCR Enzyme Mix is achieved using Mg^{2+} concentrations of 2.5 mM (as supplied). Higher Mg^{2+} concentrations will lead to lowered fidelity.
Poor performance in very long-range 2Step RT-PCR		
a)	Poor quality of cDNA template	Perform denaturation at 93°C for 15 s. Prolonged denaturation may damage template nucleic acid.
b)	Suboptimal concentration of dNTPs	Use a concentration of 500 µM of each dNTP. Increasing the dNTP concentration may negatively affect PCR results.

Appendix A: Storage, Quantification, and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A_{260}=1 → 40 µg/ml; see Tables 9 and 10). This relation is valid only for measurements at neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 35), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH,* 1 mM EDTA* followed by washing with RNase-free water. Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 20 µl of RNA sample + 180 µl buffer (1/10 dilution)

Measure absorbance of diluted sample in a 0.2 ml cuvette (RNase-free).

A_{260} = 0.2

Concentration of RNA sample	= 40 µg/ml x A_{260} x dilution factor
	= 40 µg/ml x 0.2 x 10
	= 80 µg/ml
Total amount	= concentration x volume of sample in ml
	= 80 µg/ml x 0.1 ml
	= 8 µg of RNA

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (MSDSs), available from the product supplier.

Table 9. Spectrophotometric Conversions for Nucleic Acid Templates

1 A_{260} unit*	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1

Table 10. Molar Conversions for Nucleic Acid Templates

Nucleic acid	Size	pmol/µg	Molecules/µg
1 kb DNA	1000 bp	1.52	9.1×10^{11}
2 kb DNA	2000 bp	0.76	4.6×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pBluescript® II DNA	2961 bp	0.52	3.1×10^{11}
pBR322	4361 bp	0.35	2.1×10^{11}
Lambda DNA	48,502 bp	0.03	1.8×10^{10}
Average mRNA	1930 nt	1.67	1.0×10^{12}
Genomic DNA			
Escherichia coli	4.7×10^{6}†	3.0×10^{-4}	1.8×10^{8}‡
Drosophila melanogaster	1.4×10^{8}†	1.1×10^{-5}	6.6×10^{5}‡
Mus musculus (mouse)	2.7×10^{9}†	5.7×10^{-7}	3.4×10^{5}‡
Homo sapiens (human)	3.3×10^{9}†	4.7×10^{-7}	2.8×10^{5}‡

† Base pairs in haploid genome

‡ For single-copy genes

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5.† Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1‡ in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of RNA concentration, however, we still recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA", page 33).

Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide† staining. The respective ribosomal bands (Table 11) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present at approximately twice the amounts of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

Table 11. Size of Ribosomal RNAs (rRNA) from Various Sources

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques 22, 474.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (MSDSs), available from the product supplier.

‡ Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

Appendix B: Primer Design, Concentration, and Storage

Standard PCR primers

Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. Some general guidelines are given in Table 12.

Table 12. General Guidelines for Standard PCR Primers

Length:	20–35 nucleotides
G/C content:	40–60%
T_m :	Simplified formula for estimating melting temperature (T_m): T_m = 2°C x (A+T) + 4°C x (G+C) Whenever possible, design primer pairs with similar T_m values. Optimal annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m.
Sequence:	■ Avoid complementarity of two or three bases at the 3' ends of primer pairs to reduce primer–dimer formation. ■ Avoid mismatches between the 3' end of the primer and the target-template sequence. ■ Avoid runs of 3 or more G or C at the 3' end. ■ Avoid a 3'-end T. Primers with a T at the 3' end have a greater tolerance of mismatch. ■ Avoid complementary sequences within a primer sequence and between the primer pair. ■ Commercially available computer software (e.g., OLIGO 6, Rychlik, 1999) or web-based tools such as Primer3, Steve Rosen and Helen Skaletsky, 2000 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), can be used for primer design.
Concentration:	■ Spectrophotometric conversion for primers: 1 A_{260} unit = 20–30 µg/ml

- Molar conversions:

Primer length	pmol/µg	50 pmol
20mer	152	329 ng
25mer	121	413 ng
30mer	101	495 ng
35mer	92	543 ng

- For most applications, use 1 µM of each primer (50 pmol of each primer per 50 µl reaction).

Storage: Lyophilized primers should be dissolved in a small volume of TE* (10 mM Tris·Cl, 1mM EDTA, pH 8.0) to obtain a 50 or 100 µM stock solution.

Prepare small aliquots of working solutions containing 10 µM (10 pmol/µl) to avoid repeated thawing and freezing. Store all primer solutions at –20°C. Primer quality can be checked on a denaturing polyacrylamide gel; please call one of the QIAGEN Technical Service departments or local distributor for a protocol (see back cover).

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Degenerate PCR primers

Occasionally, the exact nucleotide sequence of the target-template DNA will not be known, for instance when it has been deduced from an amino acid sequence. To enable such templates to be amplified by PCR, degenerate primers can be used. These are actually mixtures of several primers whose sequences differ at the position that correspond to the uncertainties in the template sequence. Table 13 gives recommendations for further optimizing PCR using degenerate primers. Table 14 shows the codon redundancy of each amino acid.

Table 13. Guidelines for Design and Use of Degenerate Primers

Sequence:	■ Avoid degeneracy in the 3 nucleotides at the 3' end. ■ If degeneracy cannot be avoided at the 3'-terminal bases, the oligonucleotide supplier can synthesize primers with one phosphorothioate bond between the two 3'-terminal nucleotides. ■ If possible, use Met- or Trp-encoding triplets at the 3' end. ■ To increase primer–template binding efficiency, reduce degeneracy by allowing mismatches between the primer and template, especially towards the 5' end (but not at the 3' end). ■ Try to design primers with less than 4-fold degeneracy at any given position.
Concentration:	■ Begin PCR with a primer concentration of 1 µM. ■ If PCR efficiency is poor, increase primer concentrations in increments of 0.1 µM until satisfactory results are obtained.

Table 14. Codon Redundancy

Amino acid	Number of codons
Met, Trp	1
Cys, Asp, Glu, Phe, His, Lys, Asn, Gln, Tyr	2
Ile	3
Ala, Gly, Pro, Thr, Val	4
Leu, Arg, Ser	6

Appendix C: Number of PCR Cycles

When using the LongRange 2Step RT-PCR System, a typical cycling program consists of 30–40 cycles, depending on the number of copies of the starting template and the enzyme concentration. Table 15 provides general guidelines for the selecting the appropriate number of cycles according to the number of copies of template present.

Intact RNA is essential for efficient amplification of cDNA molecules in RT-PCR. The integrity of the RNA is especially important when analyzing large fragments (e.g., amplification of complete cDNAs for protein expression). QIAGEN offers the RNeasy system for total RNA isolation and Oligotex Kits for messenger RNA isolation.*

Table 15. General Guidelines for Choosing the Number of Cycles

Number of copies of starting template†	Number of cycles
$1 \times 10^4 – 5 \times 10^4$	35–40
$5 \times 10^4 – 2 \times 10^5$	35–40
$2 \times 10^5 – 2 \times 10^6$	30–35
$2 \times 10^6 – 5 \times 10^6$	25–30

† When starting with cDNA templates, it is important to take into account the efficiency of reverse transcription in cDNA synthesis, which is on average 10–30%. Use oligo dT primers for cDNA synthesis for all PCR fragments >1 kb.

Appendix D: Gradient PCR

Many thermal cyclers have a temperature-gradient function. Using this function, it is possible to easily determine optimal annealing temperatures by generating a temperature gradient across the heating block for the annealing step. If your primers conform to the criteria specified in Appendix B on page 36, we recommend using a gradient program that includes a temperature range from 50–70°C. In order to determine optimal annealing conditions, prepare 3 identical reactions and place in the block positions that most closely correspond to the temperatures 1°C, 5°C, and 8°C below the calculated T_m of your primers.

* For further information about RNeasy and Oligotex products, contact your local QIAGEN Technical Services or distributor (see back cover). Oligotex is not available in Japan.

Appendix E: Gel Analysis of PCR Products

Analyze large amplification products on a suitable agarose gel (see Table 16), preferably in TAE-buffer, using appropriate DNA markers.

Table 16. Guidelines for Agarose Gel Analysis of Long PCR Products

Size of PCR product	Percentage of agarose	Run conditions
Up to 1 kb	1.7%	5–6 V/cm* for 0.5–1h
1–3 kb	1.5%	4–4.5 V/cm for 1–2 h
3–7 kb	1%	3–3.5 V/cm for 2h
7–15 kb	0.7%	2.5 V/cm for 5–6 h

* Voltage per cm between electrodes.

Appendix F: Purification of PCR Products

After amplification, the PCR sample contains a complex mixture of specific PCR product and residual reaction components such as primers, unincorporated nucleotides, enzyme(s), salts, mineral oil, and probably nonspecific amplification products. Before the specific PCR product can be used in subsequent experiments it is often necessary to remove these contaminants. The QIAquick® system offers a quick and easy method for purifying the final PCR product (70 bp – 10 kb). Using the MinElute® system, PCR products (70 kb – 4 kb) can be purified in higher concentrations due to the low elution volumes needed in this system. In addition, the QIAEX® II Gel Extraction Kit can be used for purification of PCR products 40 bp – 50 kb in length. Gel loading reagent and tracking dyes are effectively removed with the QIAquick and MinElute system. For more information about QIAquick and MinElute products, please call QIAGEN Technical Services or your local distributor (see back cover).

Appendix G: Control of Contamination

It is extremely important to include at least one negative control that lacks the template nucleic acid in every PCR setup to detect possible contamination.

General physical precautions

- Separate the working areas for setting up the PCR reaction mix and DNA handling, including the addition of starting template, PCR product analysis, or plasmid preparation. Ideally, use separate rooms.
- Use a separate set of pipets for the PCR reaction mix. Use of pipet tips with hydrophobic filters is strongly recommended.
- Prepare and freeze small aliquots of primer solutions and dNTP mix. Use of fresh RNase-free water is strongly recommended.
- In case of contamination, laboratory benches, apparatus, and pipets can be decontaminated by cleaning them with a 1/10 dilution of a commercial bleach solution.* Afterwards, the benches and pipets should be rinsed with RNase-free water.

General chemical precautions

- PCR stock solutions can also be decontaminated using UV light. This method is laborious, however, and its efficiency is difficult to control and cannot be guaranteed. We recommend storing solutions in small aliquots and using fresh aliquots for each PCR.
- Another approach to preventing amplification of contaminating DNA is to treat individual reaction mixtures with DNase I† or restriction enzymes† that cut between the binding sites of the amplification primers used, before adding the template DNA sample.

* Most commercial bleach solutions are approximately 5.25% sodium hypochlorate. Sodium hypochlorate is an irritant and should be handled with caution.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Appendix H: Cloning of PCR Products

PCR products contain an A overhang at the 3' end and can therefore be directly cloned into any TA- or UA-cloning vector. QIAGEN PCR Cloning Kits provide highly efficient cloning of PCR products through UA hybridization. Ligation of the PCR product, transformation, and plating of QIAGEN EZ Competent Cells takes place in just 40 minutes.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc. For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
QIAGEN LongRange 2Step RT-PCR Kit — for reverse transcription using >50 ng RNA		
QIAGEN LongRange 2Step RT-PCR Kit (20)	For 20 x 50 µl PCRs: Reverse transcription step (10 x 20 µl reactions) — LongRange Reverse Transcription Enzyme, Buffer, dNTPs, Oligo-dT, LongRange RNase Inhibitor, RNase-Free Water; PCR step — QIAGEN LongRange PCR Kit (20), see cat. no. 206401	205920
QIAGEN LongRange 2Step RT-PCR Kit (100)	For 100 x 50 µl PCRs: Reverse transcription step (50 x 20 µl reactions) — LongRange Reverse Transcription Enzyme, Buffer, dNTPs, Oligo-dT, LongRange RNase Inhibitor, RNase-Free Water; PCR step — QIAGEN LongRange PCR Kit (100), see cat. no. 206402	205922
Related products		
QIAGEN LongRange PCR Kit — for reliable and accurate long-range PCR		
QIAGEN LongRange PCR Kit (20)	For 20 x 50 µl reactions: LongRange PCR Enzyme Mix (40 U), LongRange PCR Buffer, Q-Solution, $MgCl_2$, RNase-Free Water, 10 mM dNTPs	206401
QIAGEN LongRange PCR Kit (100)	For 100 x 50 µl reactions: LongRange PCR Enzyme Mix (200 U), LongRange PCR Buffer, Q-Solution, $MgCl_2$, RNase-Free Water, 10 mM dNTPs	206402
QIAGEN LongRange PCR Kit (250)	For 250 x 50 µl reactions: LongRange PCR Enzyme Mix (500 U), LongRange PCR Buffer, Q-Solution, $MgCl_2$, RNase-Free Water, 10 mM dNTPs	206403

Ordering Information

Product	Contents	Cat. no.
HotStarTaq DNA Polymerase — for successful amplification in all applications		
HotStarTaq DNA Polymerase (250 U)*	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	203203
HotStarTaq *Plus* DNA Polymerase (250 U)*	250 units HotStarTaq *Plus* DNA Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 10x CoralLoad PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	203603
HotStarTaq Master Mix Kit (250 U)*	3x 0.85 ml HotStarTaq Master Mix containing 250 units HotStarTaq DNA Polymerase total and final concentration 1.5 mM $MgCl_2$ and 200 µM each dNTP, 2 x 1.7 ml RNase-Free water	203443
HotStarTaq *Plus* Master Mix Kit (250)*	For 250 x 20 µl reactions: 3 x 0.85 ml HotStarTaq *Plus* Master Mix, containing 250 units of HotStarTaq Plus DNA Polymerase total, 3 mM $MgCl_2$ and 400 mM each dNTP, 1 x 0.55 ml CoralLoad Concentrate, 2 x 1.9 ml RNase-Free Water	203643
QIAGEN Fast Cycling Kit — for ultrafast amplification on any thermal cycler		
QIAGEN Fast Cycling PCR Kit (200)*	For 200 x 20 µl reactions: 2 x Fast Cycling PCR Master Mix, 10 x Fast Cycling Dye, 5 x Q-Solution, RNase-Free Water	203743

* Larger kit sizes available; please inquire.

Ordering Information

Product	Contents	Cat. no.
Taq DNA Polymerase — for standard and specialized PCR applications		
Taq DNA Polymerase (250 U)*	250 units *Taq* DNA Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	201203
TopTaq™ DNA Polymerase – for highly reliable end-point PCR with unrivalled ease-of-use		
TopTaq DNA Polymerase (250)*	250 units TopTaq DNA Polymerase, 10x PCR Buffer,† 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM $MgCl_2$	200203
QIAGEN OneStep RT-PCR Kit — for easy and sensitive one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer (containing 12.5 mM $MgCl_2$), dNTP Mix (containing 10 mM each dNTP), 5x Q-Solution, RNase-free water	210210
RNeasy Kits — for purification of total RNA from animal cells or tissues, yeast, or bacteria		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Midi Kit (10)*	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75142
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-free Reagents and Buffers	75162
RNeasy Plant Mini Kit — for purification of up to 100 µg RNA from plants and fungi		
RNeasy Plant Mini Kit (20)	20 RNeasy Mini Spin Columns, 20 QIAshredder Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74903

* Larger kit sizes available; please inquire.

Ordering Information

Product	Contents	Cat. no.
QIAamp Viral RNA Mini Kit — for isolation of viral RNA from cell-free body fluids		
QIAamp Viral RNA Mini Kit (50)*	For 50 microspin viral RNA preps: 50 QIAamp Mini Spin Columns, Carrier RNA, RNase-free Buffers, Collection Tubes (2 ml)	52904
Oligotex Direct mRNA Kits — for isolation of poly A+ mRNA directly from animal cells or tissues		
Oligotex Direct mRNA Micro Kit*†	For 12 mRNA micropreps: 250 µl Oligotex Suspension, Small Spin Columns, Collection Tubes (1.5 ml), RNase-free Reagents and Buffers	72012

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

* Larger kit sizes available; please inquire.

† Not available in Japan.

Notes

Notes

Notes

www.qiagen.com

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Austria ▪ Orders 0800-28-10-10 ▪ Fax 0800/28-10-19 ▪ Technical 0800-28-10-11

Belgium ▪ Orders 0800-79612 ▪ Fax 0800-79611 ▪ Technical 0800-79556

Brazil ▪ Orders 0800-557779 ▪ Fax 55-11-5079-4001 ▪ Technical 0800-557779

Canada ▪ Orders 800-572-9613 ▪ Fax 800-713-5951 ▪ Technical 800-DNA-PREP (800-362-7737)

China ▪ Orders 86-21-3865-3865 ▪ Fax 86-21-3865-3965 ▪ Technical 800-988-0325

Denmark ▪ Orders 80-885945 ▪ Fax 80-885944 ▪ Technical 80-885942

Finland ▪ Orders 0800-914416 ▪ Fax 0800-914415 ▪ Technical 0800-914413

France ▪ Orders 01-60-920-926 ▪ Fax 01-60-920-925 ▪ Technical 01-60-920-930 ▪ Offers 01-60-920-928

Germany ▪ Orders 02103-29-12000 ▪ Fax 02103-29-22000 ▪ Technical 02103-29-12400

Hong Kong ▪ Orders 800 933 965 ▪ Fax 800 930 439 ▪ Technical 800 930 425

Ireland ▪ Orders 1800 555 049 ▪ Fax 1800 555 048 ▪ Technical 1800 555 061

Italy ▪ Orders 800-789-544 ▪ Fax 02-334304-826 ▪ Technical 800-787980

Japan ▪ Telephone 03-6890-7300 ▪ Fax 03-5547-0818 ▪ Technical 03-6890-7300

Korea (South) ▪ Orders 080-000-7146 ▪ Fax 02-2626-5703 ▪ Technical 080-000-7145

Luxembourg ▪ Orders 8002-2076 ▪ Fax 8002-2073 ▪ Technical 8002-2067

Mexico ▪ Orders 01-800-7742-639 ▪ Fax 01-800-1122-330 ▪ Technical 01-800-7742-436

The Netherlands ▪ Orders 0800-0229592 ▪ Fax 0800-0229593 ▪ Technical 0800-0229602

Norway ▪ Orders 800-18859 ▪ Fax 800-18817 ▪ Technical 800-18712

Singapore ▪ Orders 1800-742-4362 ▪ Fax 65-6854-8184 ▪ Technical 1800-742-4368

Spain ▪ Orders 91-630-7050 ▪ Fax 91-630-5145 ▪ Technical 91-630-7050

Sweden ▪ Orders 020-790282 ▪ Fax 020-790582 ▪ Technical 020-798328

Switzerland ▪ Orders 055-254-22-11 ▪ Fax 055-254-22-13 ▪ Technical 055-254-22-12

UK ▪ Orders 01293-422-911 ▪ Fax 01293-422-922 ▪ Technical 01293-422-999

USA ▪ Orders 800-426-8157 ▪ Fax 800-718-2056 ▪ Technical 800-DNA-PREP (800-362-7737)

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